Studies on the regulation of IgE.

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Studies on the Regulation of Immunoglobulin E

by

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ABSTRACT

The allergic response is mediated through the ligand-receptor pair immunoglobulin E (IgE), produced by B lymphocytes, and the high affinity receptor for IgE (FcεRI), present on certain myeloid cells. When IgE is attached to the receptor it can bind allergen, which cross-links receptors on the cell surface, causing the release of histamine, prostaglandins, cytokines, and other mediators of the allergic reaction, resulting in among other effects, the up-regulation of IgE levels. A number of important events in the regulation of IgE were studied, including the cloning and characterisation of the gene for the FcεRIα subunit, the analysis of a switch recombination event to IgE, the cloning, expression, and regulation of the germline gene transcript for IgE, and the mechanism of glucocorticoid enhancement of IgE secretion.

Due to its important role in the transduction of signals involved in the allergic reaction, the gene for the IgE binding subunit FcεRIα was cloned and its structure analysed. A promoter fragment was identified that contained a C/T polymorphism at a potential GATA transcription factor binding site that may be linked to allergy. All 4 of the individuals containing a C allele were non-atopic, from a group of 15 atopics and 15 non-atopics.

IgE is expressed by B cells after an isotype switching event from IgM, or another isotype. To determine if switching to IgE corresponded to a DNA recombination event in the immunoglobulin heavy chain locus, the region upstream of the epsilon locus was sequenced in a B cell line, U266, that underwent switching to IgE. U266 was shown to be the result of a IgM→IgA1→IgE series of switch recombination events and to be the first example of sequential switching.

Prior to switch recombination, transcription (germline gene transcription) occurs through the mu locus and within the constant region locus to which switching is directed. The germline gene transcript (GLT) for IgE was cloned, and its expression was found to precede the secretion of IgE in peripheral blood mononuclear cells (PBMC). ε-GLT were detected 12 hours after IL-4 induction, reaching a maximum at 3 days. Transcription start sites were heterogeneous within a 220 base region upstream of the germline exon. A 650 bp fragment was found to have promoter activity in B lymphoblastoid lines and to bind the transcription factor BSAP in vitro. BSAP was found to be required for the IL-4 and CD40 mediated up-regulation of transcription and is thought to be responsible for its B cell specific expression.

Glucocorticoids (GC) provide a co-stimulus to purified B cells to switch to IgE in the presence of IL-4. GC also enhance IgE synthesis *in vivo*, and in PBMC. Surprisingly, GC are the main pharmacological agent used to control the symptoms of allergy. The mechanism of the GC potentiation of IgE synthesis in PBMC was investigated to elucidate this potentially antagonistic phenomenon. IgE enhancement was found to occur only after 10 days of glucocorticoid addition in *vitro*. Germline gene and productive epsilon transcription was not enhanced in the presence of IL-4 by glucocorticoid at 4.5 days of culture, indicating that glucocorticoids could act on switching though a unique mechanism later in the culture period.

Confirmation of the linkage of the FcεRIα polymorphism to allergy could lead to the discovery of transcription factors that bind to this sequence which could be targets for anti-allergy strategies. Further work on the role of the ε-GLT, and the structure of the switch region in the mechanism of switching could help to clarify the role of transcription and chromatin structure in recombination. A knowledge of the mechanism of GC potentiation of IgE secretion could lead to new IgE regulatory routes, and help improve the efficacy of glucocorticoids in the treatment of allergy.

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To Cole.

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LIST OF ABBREVIATIONS

3' LCR	3' immunoglobulin locus control region
AP-1	Activating Protein-1
β-gal	β-galactosidase
bHLH	basic-helix-loop-helix
bp	base pair(s)
BSA	Bovine serum albumin
BSAP	B cell lineage-specific activator protein
C region	Immunoglobulin heavy chain constant region
C/EBP	CCAAT/enhancer-binding protein
CBP	CREB-binding protein
CD23	Low affinity receptor for IgE
CD40	Cluster designation 40
CD40-L	CD40-Ligand
C_H	Immunoglobulin heavy chain constant region
CREB	cAMP response element binding protein
DEAE	Diethylaminoethyl cellulose
DEPC	Diethyl pyrocarbonate
dH_2O	Water from Milli-Q UF Plus (Millipore, Bedford, MA) deioniser.
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra-acetic acid
ε-GLT	epsilon germline gene transcription
ELISA	Enzyme-linked immunoabsorbent assay
Eμ	immunoglobulin J_H-Cμ enhancer
EMSA	Electrophoretic mobility shift assay, move-up, band-shift
Fc	Immunoglobulin constant region fragment
FcεRI	High affinity receptor for IgE
FcεRIα	High affinity receptor for IgE alpha chain
FcεRII	Low affinity receptor for IgE
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GITC	Guanidine isothiocyanate
GL-ε	Germline-epsilon
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HMG-I(Y)	High mobility group-I(Y)
I exon	"Intronic" (germline gene) exon
IAPP	Islet amyloid polypeptide
IgE	Immunoglobulin E
IgH	Immunoglobulin heavy chain
IL-4	Interleukin-4
IPTG	Isopropylthio-β-galactoside
JNK	c-Jun N-terminal kinase cascade
LCR	locus control region
M-MLV RT	murine-moloney leukaemia virus reverse transcriptase
NF-HB	B-lineage-specific nuclear factor that binds to Ig H gene segments
NF-κB	Nuclear factor-kappa B
NH4+	Ammonia
NH4OAc	Ammonium acetate
NP	Blood donor Nick Powell
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEG	Polyethelene glycol
PFU	Plaque forming units
PMSF	Phenylmethylsulphonyl flouride
RT-PCR	Reverse transcription PCR
S region	Switch region
SD	Blood donor Steven Durham
SDS	Sodium dodecyl sulphate
Speedvac	Centrifuge in a "Speedvac" concentrator (Savant, Hicksville NY)
STAT	Signal transducer and activator of transcription
T4	bacteriophage T4

TBE	Tris borate gel running buffer
TE	10mM Tris-HCl pH 8, 0.1 mM EDTA
TH	T helper
TNF-R	Tumour necrosis factor-receptor
TRAFs	TNF-R-associated factors
Tris	Tris[hydroxymethyl]aminomethane
TW	Blood donor Timothy Whitfield
Tween 20	Polyoxyethylenesorbitan monolaurate
Tween 80	Polyoxyethylenesorbitan monooleate
USF	Upstream stimulatory factor
VDJ-Cε	VDJ containing (productive) IgE transcript
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside
YACs	Yeast artificial chromosomes

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CHAPTER 1: Introduction

1.1 Antibody structure, function and expression.

During an immune response, antibodies specific to a foreign antigen are produced by B cells. Antibodies are composed of four polypeptide chains, two light chains, and two heavy chains, encoded on two genes (for review see Blackwell and Alt 1988; Casali and Schettino 1996). Antigen binding occurs via the variable region at the amino terminus, and effector functions occur via the constant region (Fig. 1.1). In humans, five classes (isotypes) of antibodies are produced, IgM, IgD, IgG, IgE, and IgA, and six subclasses, IgG1-IgG4 and IgA1 and 2, all differing at their heavy chain constant region (C_H). After heavy chain variable region gene assembly, the antibody IgM is expressed in the cytoplasm (Fig. 1.2). The presence of cytoplasmic mu chain signals light chain variable region assembly, allowing production of light chain protein and the expression of an IgM molecule on the cell surface. Initially IgM, and by alternative splicing IgD, are produced by the B cell. Later, after antigen is encountered, the effector functions of the antibody can change in a class switching event that replaces the μ, or δ constant region with that of γ, ε, or α. Class switching allows the IgG, IgE, and IgA isotypes to be produced without changing the variable region expressed.

IgM comprises the initial rapid antibody response in the blood, and at sites of infection after vascular permeability is increased. IgD is expressed on the surface of mature B cells and may be involved in B cell activation (Finkelman *et al.* 1992). IgG is the principal antibody of the blood and extracellular fluid. IgA is the principal isotype in secretions, most importantly the mucus membranes of the intestinal, and respiratory tracts. IgE is produced in low amounts in peripheral blood or extracellular fluid. IgE is involved in the cell mediated killing of parasitic worms (helminths) (Butterworth *et al.* 1992; Capron *et al.* 1992; Gounni *et al.* 1994), and mediates allergic disease.

1.2 The immunological basis of allergic disease.

Helminth infection affects two thirds of the world's population, and in some tropical areas, chronic schistosomiasis is the most pressing health concern. Allergic disease affects about 20 percent of the world's population (Casolaro *et al.* 1996), and because of its prevalence in the

western world, most of the research on IgE has been from the viewpoint of allergy. The nature of the IgE directed response to helminth infection, and its role in allergy are quite different. Although both allergy and helminth resistance share the use of the Th2 cytokines interleukin-4 and interleukin-5 (IL-4, IL-5), (Coffman *et al.* 1989; Thyphronitis *et al.* 1993; Bressler *et al.* 1997), in other respects the two functions appear to use different pathways. Resistance to helminth infection exhibits both a combined Th1/Th2 response, though in IL-4 deficient mice survival of the filarial nematode *Brugia malayi* is unaffected despite the lack of a Th2 pathway (Lawrence *et al.* 1995). Knockouts of the high affinity receptor for IgE alpha chain (FcεRIα) are resistant to anaphylaxis, but respond normally to helminth infection (Dombrowicz *et al.* 1993; Jankovic *et al.* 1997), indicating that interactions through the high affinity receptor is required for allergy but not helminth resistance. Curiously, individuals chronically infected with intestinal nematodes have less dermal allergic reactivity to common allergens than helminth-free individuals in the same environment (Lynch *et al.* 1993). One explanation for this phenomenon is that IgG4 levels are high in chronically infected individuals (Jassim *et al.* 1987; Hagan *et al.* 1991; Demeure *et al.* 1993). High IgG4 levels have been correlated with a resistance to allergic symptoms acting through a proposed antagonism between IgG4 and IgE (Hagan *et al.* 1991; Rihet *et al.* 1992; Demeure *et al.* 1993).

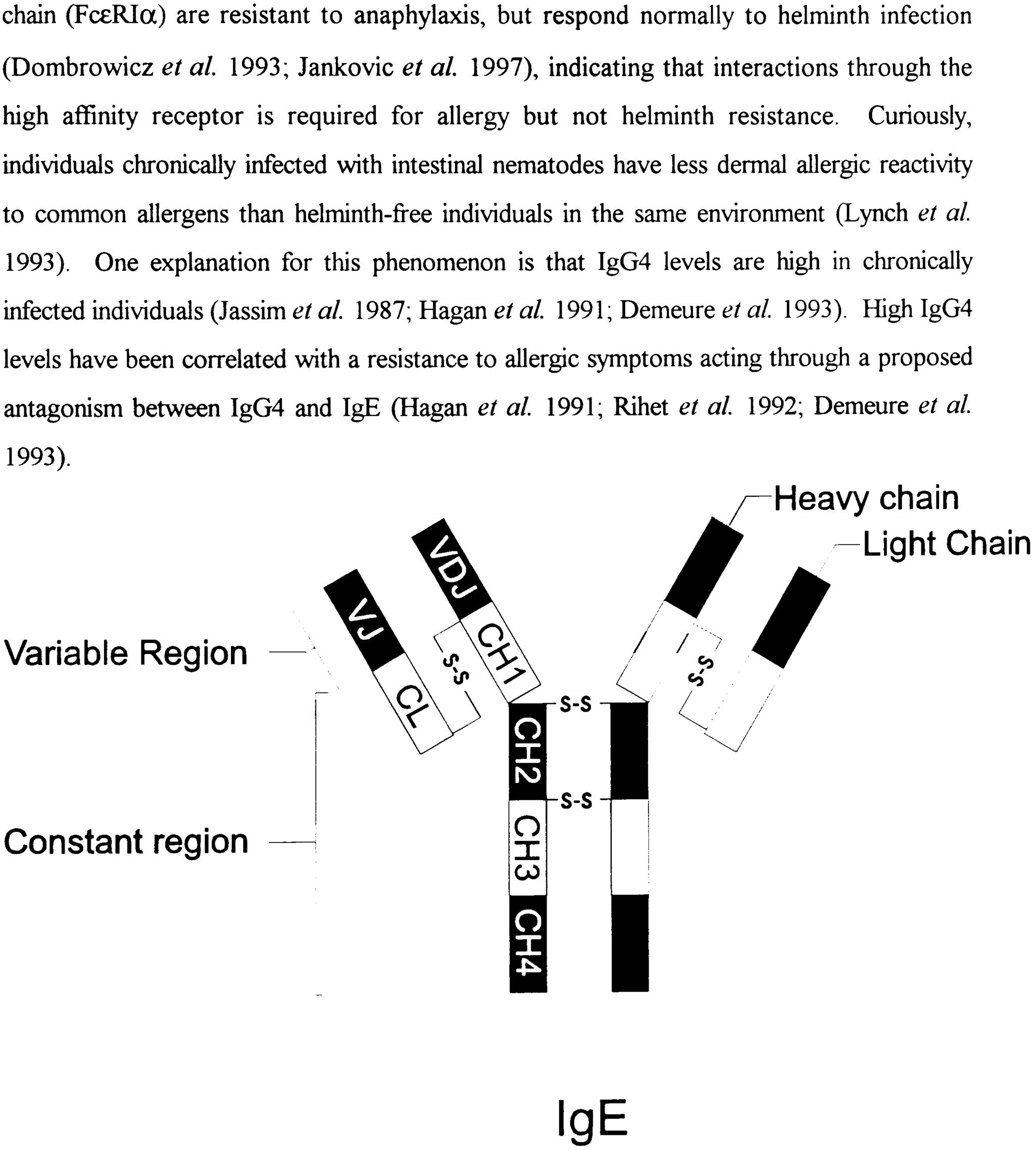


Figure 1.1. Structure of an antibody. IgE is given as an example antibody. Four polypeptide chains, two heavy and two light are held together by disulfide bridges (S-S).

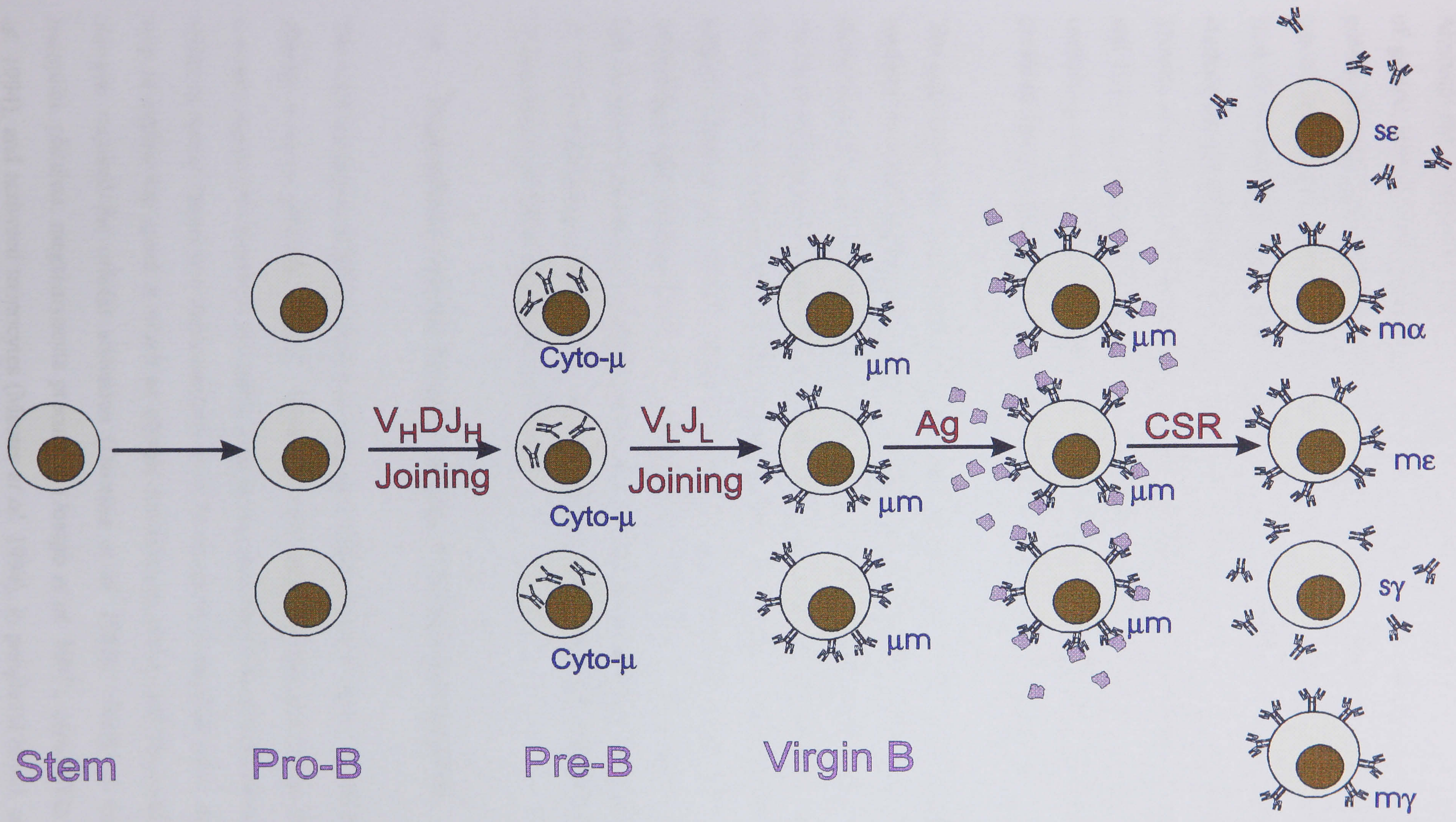


Figure 1.2. Immunoglobulin expression during B cell development. Cyto, cytoplasmic. Ag (purple), antigen. CSR, class switch recombination. m, membrane immunoglobulin. s, secreted immunoglobulin.

1.3 The genetic basis for allergy.

Although the causes of allergy are not known, much circumstantial data and a small amount of genetic linkage data have lead to some theories. Present evidence suggests multiple genetic loci are responsible for a predisposition to allergy (Bleecker *et al.* 1997). Several reports have shown a genetic linkage to serum IgE levels at chromosome 5q31-q33 near the IL-4, IL-13 and IL-9 genes, at chromosome 11q13 near the FcεRIβ gene, and at several markers on chromosome 12q (Marsh *et al.* 1994; Doull *et al.* 1996; Noguchi *et al.* 1997; Thomas *et al.* 1997). Other reports have not been able to confirm the linkage to 5q31-q33 and 11q13 to serum IgE levels or asthma, (Laitinen *et al.* 1997; Thomas *et al.* 1997). The conflicting results could be due to differences in the populations studied, combined with the predicted low percentage of atopics that would correspond to these mutations.

The type 2 cytokine locus appears to have the strongest genetic linkage to allergy. IL-4 is required for switching to IgE *in vitro* (Vercelli and Geha 1992; Coffman *et al.* 1993), is elevated in the blood of some atopics (Matsumoto *et al.* 1994; Ohshima *et al.* 1995), and in the local environment of the nasal epithelium after allergen challenge (Durham *et al.* 1997). The T cells of atopics also produce higher amounts of IL-4 after stimulation than normal subjects (Pene *et al.* 1994; Stanciu *et al.* 1997). IL-4 knockout mice are defective in switching to IgE (Kuhn *et al.* 1991; Lawrence *et al.* 1995). IL-13 also induces switching to IgE *in vitro* (Punnonen *et al.* 1997), is secreted in response to allergen challenge (Huang *et al.* 1995; Gabrielsson *et al.* 1997), and is elevated in the bronchial epithelium of atopics (Kotsimbos *et al.* 1996; Humbert *et al.* 1997).

1.4 High affinity receptor for IgE structure, function and expression.

The constant region of IgE binds to two receptors, a high affinity receptor (FcεRI) and a low affinity receptor (FcεRII, CD23). Unlike all other isotypes, IgE can remain bound to its constant region (Fc) receptors in a stable, long lasting complex in the absence of antigen. The ability to remain bound in a stable complex to its Fc receptors in the absence of antigen might help to explain the speed at which an allergic reaction can occur, and the small amount of allergen required for cellular activation (Davenas *et al.* 1988). FcεRI is expressed on basophils, platelets, megakaryocyte precursors (Joseph *et al.* 1997), eosinophils (Gounni *et al.* 1994), and activated monocytes (Maurer *et al.* 1994), in peripheral blood, and on mast

cells, and Langerhans cells in tissue (Bieber *et al.* 1992; Wang *et al.* 1992). FcεRI is composed of 3 subunits; α, β and γ2 (Fig. 1.3). The α subunit binds to IgE, the β subunit is involved in amplifying the signal transduction functions of the γ chains (Lin *et al.* 1996). The β chain is not expressed on Langerhans cells, suggesting it is not crucial for receptor function (Jurgens *et al.* 1995).

Little is known about the regulation of the high affinity receptor for IgE. The expression of FcεRI is regulated by some of the stimuli that control the allergic reaction and helminth resistance. Namely, IL-4 stimulates FcεRI expression in cord blood mast cells (Toru *et al.* 1996), and nasal mast cells (Pawankar *et al.* 1997). IgE also regulates FcεRI expression (Lantz *et al.* 1997; Yamaguchi *et al.* 1997), suggesting receptor levels are regulated to accommodate increases in IgE during an immune reaction. As more becomes known about the regulation of the high affinity receptor, strategies to control its expression may provide new therapies for allergy.

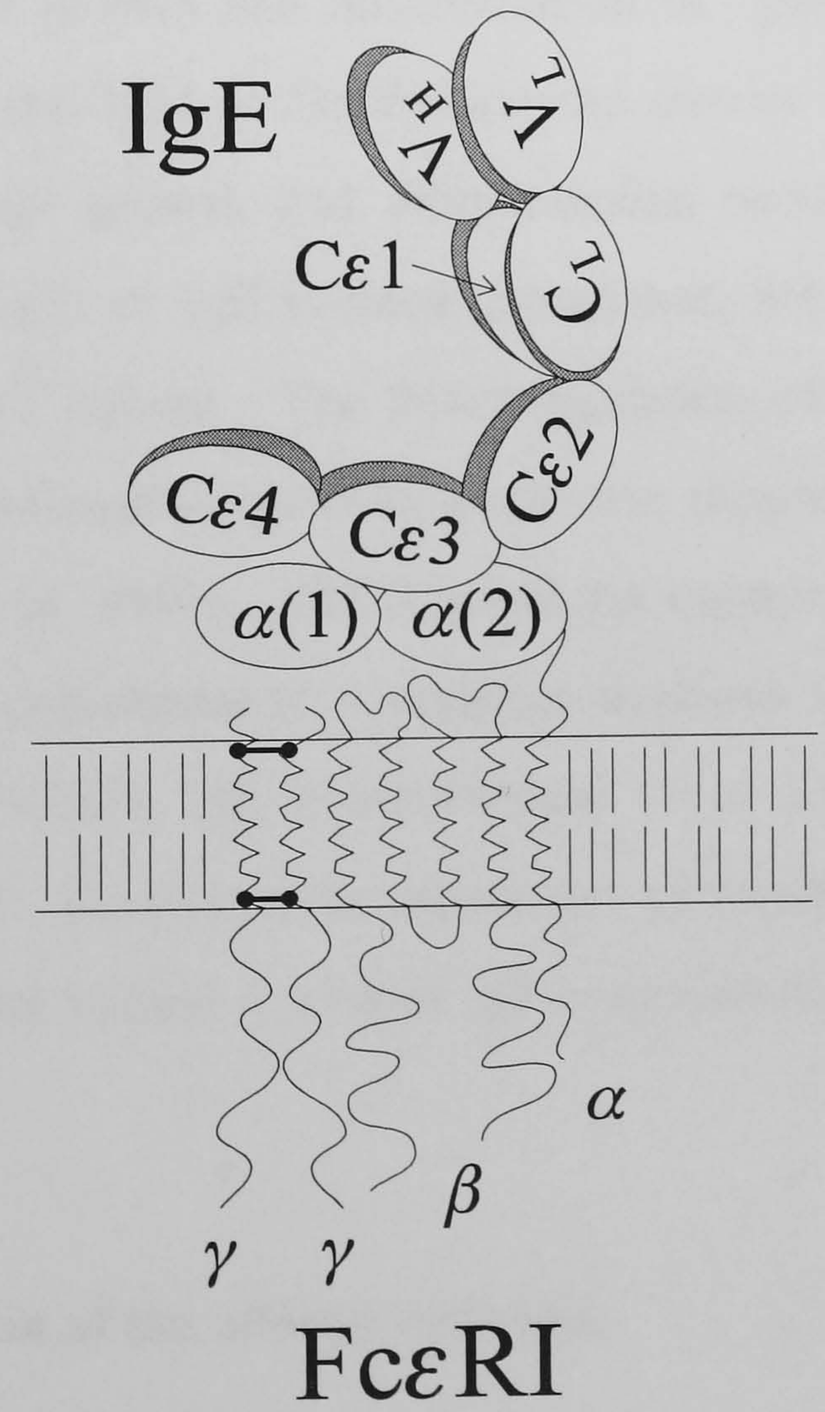


Figure 1.3. Structure of the high affinity receptor for IgE. The immunoglobulin domains α(1) and α(2) of the high affinity receptor alpha chain bind the constant region of IgE. Each of the α, β and γ_2 chains of the receptor contain trans-membrane portions.

1.5 The low affinity receptor for IgE: A dual regulatory role.

CD23 exists in both a membrane bound form, and as soluble fragments, though the two molecules have strikingly different roles in the regulation of IgE (for review see Gould *et al.* 1997). sCD23 up-regulates both ongoing spontaneous IgE from atopic patients (Sarfati *et al.* 1984), and acts synergistically with sub-optimal IL-4 concentrations to induce IgE synthesis in human B cells (Henchoz *et al.* 1994). An antibody against CD21, which is known to interact with CD23, provides the same co-signal to B cells, suggesting that the stimulus from sCD23 is transduced through the CD21 molecule (Henchoz *et al.* 1994).

Cross-linkage of membrane CD23 has the opposite effect of sCD23 on IgE secretion, down-regulating spontaneous IgE production by PBMC from atopic (Sherr *et al.* 1989; Sarfati *et al.* 1990), and inhibiting the IL-4 induced IgE synthesis from normal PBMC (Pene *et al.* 1988; Sarfati and Delespesse 1988). Cross-linkage of membrane CD23 also caused an inhibition of growth and differentiation of peripheral blood B cells in the presence of IL-4, and anti-IgM or *Staphylococcus aureus* Cowan A (Luo *et al.* 1991). The inhibition of B cell growth and differentiation occurred whether by anti-CD23 antibodies, polymeric IgE, or IgE immune complexes, but the cross-linking agent was required at the onset of culture. The down-regulation of ongoing IgE synthesis in B lymphoblastic lines was found to occur by a selective suppression of the secreted form of IgE mRNA (Saxon *et al.* 1991). CD23 knockout experiments support the conclusion that membrane CD23 contributes to a negative feedback route for IgE, demonstrating that in the absence of CD23, IgE levels increase 10 to 100-fold higher than wild-type mice (Yu *et al.* 1994). Further, over-expression of membrane CD23, but not soluble CD23 in transgenic mice caused a weaker IgE response than wild-type mice (Texido *et al.* 1994).

1.6 The progression of the allergic reaction.

Upon contact with allergen both an immediate and a delayed hypersensitivity reaction occur. Immediate hypersensitivity occurs within minutes and is initiated when IgE bound to a mast cell or basophil via its high affinity receptor recognises an allergen (for review see Beaven and Metzger 1993; Paul *et al.* 1993; Sutton and Gould 1993). Cross-linking of the high affinity

receptor on a mast cell or basophil causes cellular activation and the exocytosis of intracellular granules (Fig. 1.4), which contain numerous preformed intracellular mediators of the allergic reaction (Galli *et al.* 1993). Mast cell and basophil degranulation releases histamine, which causes inflammation and swelling, neutral proteases, which have a variety of roles in inflammation, and proteoglycans which serve to facilitate the uptake and packaging of the other preformed mediators including histamine and proteases (Redington *et al.* 1995; Metcalfe *et al.* 1997). In addition to preformed mediators released from granules, mast cell activation also causes the release of several classes of mediators synthesised *de novo* from membrane lipids, including prostaglandins and leukotrienes, which are involved in inflammation and swelling (Redington *et al.* 1995; Metcalfe *et al.* 1997). Cytokines are also released during degranulation, and produced *de novo* after mast cell activation, including TNF-α, IL-4, and IL-5 each of which play a role in many events involved in delayed hypersensitivity (Heusser *et al.* 1991; Walsh *et al.* 1991; Galli *et al.* 1993; Metcalfe *et al.* 1997).

Delayed hypersensitivity occurs through cellular interactions which are only recently becoming known. IL-4 recruits eosinophils and lymphocytes through the up-regulation of cellular adhesion molecules (Metcalfe *et al.* 1997). Similarly, IL-5 is responsible for eosinophil chemotaxis, proliferation and maturation (Walsh *et al.* 1991; Metcalfe *et al.* 1997). IL-4 also up-regulates acquisition of a Th2 cytokine profile in T cells

When FcεRI is cross-linked on the surface of dendritic cells such as Langerhans cells, the allergen is presented to T cells (Bieber 1997; Stingl and Maurer 1997), up-regulating both immediate and delayed hypersensitivity reactions. B cells can also present allergen to T cells through its binding to the low affinity receptor for IgE (Pirron *et al.* 1990), resulting in T cell activation, up-regulating both immediate and delayed hypersensitivity reactions (Fig. 1.4). The presentation of allergen to T cells by both dendritic cells and B cells potentially results in the up-regulation of immunoglobulin class switching events from IgM, IgG_{1-4}, or IgA_1 to IgE (Grewal and Flavell 1996; Mudde *et al.* 1996).

Anti-allergy strategies involving disruption of the interaction between IgE and both the high and low affinity receptors appear to act through an inhibition of antigen presentation by B cells. Humanised antibodies against the high and low affinity receptor binding sites on IgE have been demonstrated to reduce basophil FcεRI expression, serum IgE levels, inhibit antigen induced skin reactions, reduce rhinitis symptoms and early and late phase bronchoconstriction responses (Heusser and Jardieu 1997; MacGlashan *et al.* 1997). The suppression of allergen induced IL-4 and IL-5 by production T cells and eosinophil infiltration to the lung by the anti-IgE antibodies is maintained in mast cell deficient mice, suggesting that inhibition of the CD23-IgE facilitated antigen presentation to T cells is at least one mechanism (Coyle *et al.* 1996). Similarly, FcεRIα peptide fragments corresponding to the IgE binding site also inhibit mast cell degranulation in vitro and IgE mediated graft-versus-host disease in mice (McDonnell *et al.* 1996; Korngold *et al.* 1997), through competition with the high affinity receptor for IgE binding.

IL-4, and potentially IL-5, released from activated mast cells up-regulate immunoglobulin class switching to IgE (Vercelli *et al.* 1989; Purkerson and Isakson 1992). TNF-α, also released after mast cell activation, acts synergistically on the IL-4 induction of IgE synthesis from human B cells (Gauchat *et al.* 1992).

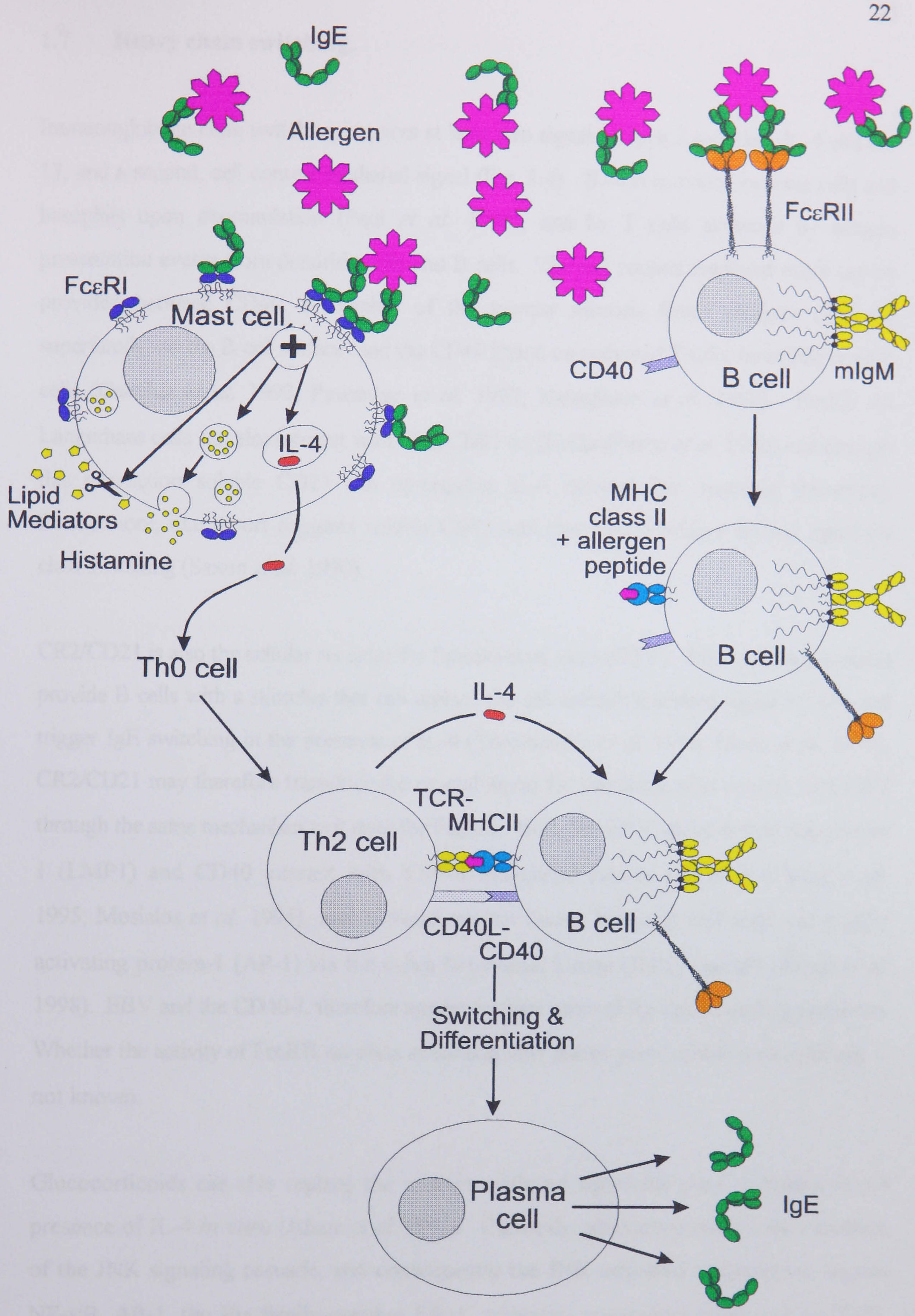


Figure 1.4. The role of mast cell activation and antigen presentation by B cells in switching to IgE. Allergen (purple) is processed by B cells and presented to T cells in the context of class II MHC, up-regulating heavy chain switching to IgE. The cross-linking of FcεRI on the surface of mast cells causes the release IL-4, which is important for the differentiation of T cells to the Th2 phenotype and as a co-stimulus for switching to IgE.

1.7 Heavy chain switching.

Immunoglobulin class switching requires at least two signals, a type 2 cytokine, IL-4 and IL-13, and a second, cell contact mediated signal (Fig. 1.4). IL-4 is provided by mast cells and basophils upon degranulation (Paul *et al.* 1993), and by T cells activated by antigen presentation events from dendritic cells and B cells. The cell contact mediated event can be provided between CD40, a member of the tumour necrosis factor receptor (TNF-R) superfamily, on the B cell surface and the CD40 ligand on activated T cells, basophils or mast cells (Gauchat *et al.* 1993; Pawankar *et al.* 1997; Yanagihara *et al.* 1998). FcεRII on Langerhans cells can also interact with CR2/CD21 on B cells (Pirron *et al.* 1990) and through this interaction soluble CD23 can up-regulate IL-4 induced IgE synthesis (Henchoz). Furthermore, one report suggests soluble CD23 and may also provide a second signal for class switching (Saxon *et al.* 1990).

CR2/CD21 is also the cellular receptor for Epstein-Barr virus (EBV). EBV encoded proteins provide B cells with a stimulus that can replace the cell contact mediated signal *in vitro* and trigger IgE switching in the presence of IL-4 (Thyphronitis *et al.* 1989; Jabara *et al.* 1990). CR2/CD21 may therefore transduce the second signal for switching after contact with EBV through the same mechanism as it does for FcεRII. Both the EBV latent membrane protein 1 (LMP1) and CD40 interact with TNF-R-associated factors (TRAFs) (Cheng *et al.* 1995; Mosialos *et al.* 1995), and activate nuclear factor kappa B (NF-κB) and trigger activating protein-1 (AP-1) via the c-Jun N-terminal kinase (JNK) cascade (Kilger *et al.* 1998). EBV and the CD40-L therefore appear to share some of the same signaling pathways. Whether the activity of FcεRII on class switching also shares parts of the same pathway is not known.

Glucocorticoids can also replace the contact mediated signal for class switching in the presence of IL-4 *in vitro* (Jabara *et al.* 1991). Curiously, glucocorticoids block induction of the JNK signaling cascade, and consequently the JNK activated transcription factors NF-κB, AP-1, the Ets family member Elk-1, activating transcription factor-2 (ATF-2), and CREB-binding protein (CBP) recruitment (Adcock *et al.* 1995; Caelles *et al.* 1997). Glucocorticoids interfere directly with NF-κB and AP-1 function through the physical interactions with the glucocorticoid receptor (Jonat *et al.* 1990; Schule *et al.* 1990;

Yang-Yen *et al.* 1990; Ray and Prefontaine 1994; Barnes 1996; De Bosscher *et al.* 1997; Liden *et al.* 1997), and up-regulate IκB synthesis (Auphan *et al.* 1995). The interference with NF-κB function and the block of the JNK pathway by glucocorticoids is in apparent contradiction to the role of NF-κB and the JNK cascade in the EBV/CD40-L induced cell contact mediated signal. The mechanism of action of glucocorticoids in replacing the second signal is hence likely to be of a different nature than the EBV or CD40-L mechanism, such as through the direct transactivating potential of the glucocorticoid receptor on many genes (for review see McEwan *et al.* 1997).

The human immunoglobulin heavy chain locus is composed of about 1100 kb of variable region gene segments upstream of 350 kb of constant gene segments arranged μ, δ, γ3, γ1, εψ, α1, γ2, γ4, ε, α2 (Hofker *et al.* 1989; Walter *et al.* 1990) (Fig. 1.5). The heavy chain components of IgM and IgD are produced by alternatively spliced transcripts initiating at the V region promoter upstream of the rearranged variable region. Immunoglobulin class switching to IgG, IgA, or IgE requires a DNA recombination event between a switch (S) region sequence upstream of the mu constant region and a S region upstream of the isotype to which switching is directed.

Switch regions contain repeating pentameric units which may promote the pairing of the two switch regions undergoing recombination by enhancing homology between the two sequences (Fig. 4.5). Switch regions fall into two homology groups. Sμ, Sε, and Sα form the first group, sharing considerable homology and containing the main pentameric units GAGCT and GGGCT (Mills *et al.* 1990). The $S\gamma_{1\text{-}4}$ regions form the second group containing the main pentameric units GCAGG and CAGGG (Thienes and Gould unpublished observations). The role of the pentameric repeats and switch region topology in the mechanism of heavy chain recombination remains largely unknown.

1.8 The regulation of transcription.

Gene expression may be regulated at several levels: the activation of chromatin structure, initiation of transcription, processing of the transcript, mRNA stability, transport of the transcript to the cytoplasm, translation of mRNA, post-translational modification, transport of the protein to the nucleus, and the stability of the protein.

Eukaryotic genes typically have proximally located transcriptional control elements, comprising the promoter, and additional distal elements which regulate the chromosomal domain. Genes are regulated on the transcriptional level at the promoter by two types of factors. First, general factors bind to RNA polymerase to assemble the pre-initiation complex, which forms around the start site of transcription, and is required for the initiation of transcription (for transcription review, see Orphanides *et al.* 1996; Roeder 1996; Lewin 1997). Upstream factors, both inducible and constitutive, compose the second group and modulate the efficiency of initiation.

Distal elements include enhancers and locus control regions (LCRs) which typically increase the likelihood of gene expression, silencers which repress expression, and insulators which limit control to an individual locus. Enhancer elements act to increase the activity of promoters at great distances from the start site of transcription (usually several kb from the promoter), can be located upstream, downstream or within the transcriptional unit, and function in either orientation relative to the start site of transcription. Enhancers potentiate activity from the promoter by acting to increase the probability of expression in a given cell, though not increasing the level of expression per cell (Martin *et al.* 1996). A locus control region (LCR), is often found at the border of a gene locus, affecting expression of the gene in a position independent and copy number dependent manner. The LCR is thought to act by exerting dominant transcriptional activating or inactivating functions over an entire chromatin domain by communication with enhancers and promoters and/or by modifications of chromatin accessibility (Martin *et al.* 1996).

The transcriptional activity of genes is influenced by not only the availability of transcription factors, but also the access to chromatin for these factors. When the DNA template is wrapped around histone proteins to form nucleosomes, factor access is restricted. Acetylation of the core histones, causing destabilisation of nucleosomes, is associated with transcriptional activity (for review see Struhl 1998). Proteins associated with histone acetyltransferase and histone acetylase (deacetylase) activity have been shown to participate in the activation and repression of transcriptional activity, respectively. Many of the

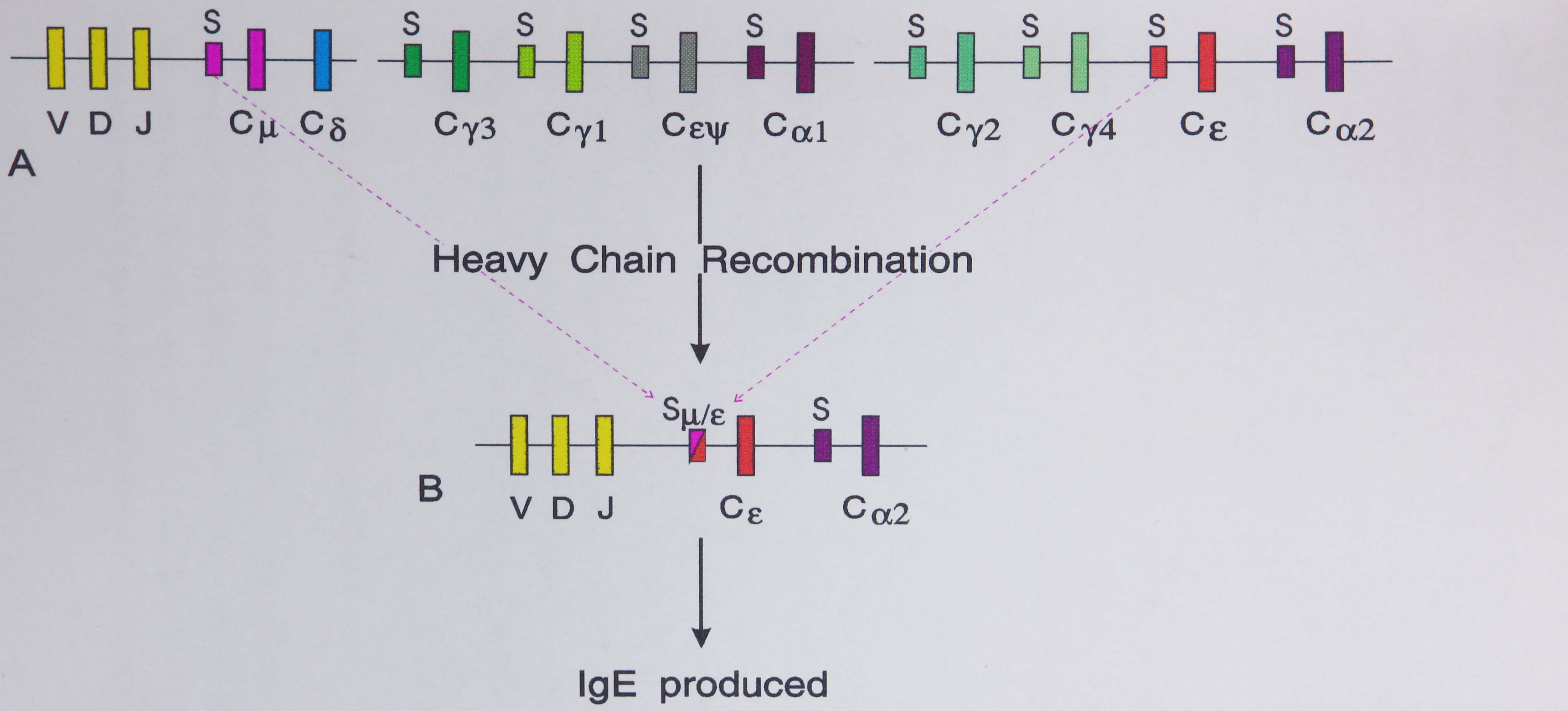


Figure 1.5. Heavy chain recombination. (*A*) The immunoglobulin heavy chain locus. VDJ, the rearranged variable region genes. C_x, the heavy chain constant region loci, and S the switch regions upstream of each constant region gene segment. Switching occurs by a recombination event between the mu switch region, and a downstream switch region. (*B*) Recombination generates a deletion of the intervening constant region loci, juxtaposing a new constant region next to the variable region gene segments and promoter.

acetyltransferase and acetylase activities described have been shown to be associated with the basal transcription machinery, as factors previously described as transcriptional coactivators and corepressors, respectively. The TAF130/250 histone acetyltransferase is a subunit of the TFIID complex, a basic component of the DNA polymerase II (Pol II) transcription machinery (Mizzen *et al.* 1996). The coactivator p300/CBP histone acetyltransferase is associated with the Pol II holoenzyme (Nakajima *et al.* 1997).

One of the most intensively studied family of transcription factors associated with acetyltransferase and acetylase activities are the nuclear-hormone receptors. The progesterone receptor (PR), glucocorticoid receptor (GR), estrogen receptor (ER), thyroid hormone receptor (TR), and the retinoic acid receptor (RXR) bind to the histone acetyltransferase steroid receptor coactivator-1 (SRC-1) (Onate *et al.* 1995). Similarly, a second nuclear receptor coactivator, ACTR, has also been shown to possess acetyltransferase activity (Chen *et al.* 1997). Both SRC-1 and ACTR recruit the acetyltransferases CBP/P300 and P/CAF thereby forming protein complex containing multiple histone acetyltransferases (Chen *et al.* 1997; Spencer *et al.* 1997). In the absence of hormone, TR and TR/RXR heterodimers bind corepressors which can be dissociated by thyroid hormone in the presence of coactivators (Olson *et al.* 1998). Interaction of the corepressors silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (N-CoR) with the nuclear receptor associates with mammalian Sin3a, which in turn recruits the histone deacetylase HDAC1 to the receptor/corepressor complex (Alland *et al.* 1997; Nagy *et al.* 1997).

1.9 The regulation of heavy chain recombination.

Recombination in the heavy chain locus requires at least two locus control elements, the immunoglobulin J_H-Cμ enhancer (Eμ) in the J_H-Cμ intron (Banerji *et al.* 1983; Gillies *et al.* 1983; Mercola *et al.* 1983), and the 3' immunoglobulin locus control region (LCR) downstream of the Cα gene in the mouse (Madisen and Groudine 1994), and both Cα

genes in the human (Mills *et al.* 1997). Deletion of Eμ significantly reduces V region recombination (Chen *et al.* 1993; Serwe and Sablitzky 1993), and recombination within the mu switch region (Gu *et al.* 1993). In Eμ deficient mice recombination occurs within and between downstream switch regions, though because recombination does not involve Sμ, these are not productive switching events (Gu *et al.* 1993). Mutation of the 3' LCR affects recombination in the downstream switch regions (Cogne *et al.* 1994), but does not affect variable region assembly or recombination within Sμ.

Prior to switch recombination, transcription occurs through the mu locus and within the constant region locus to which switching is directed (for review see Vercelli and Geha 1992; Coffman *et al.* 1993). In each case transcription (germline transcription) begins in a short exon (germline exon, I exon) upstream of the switch region and proceeds through the constant region (Fig 1.6). The germline exon contain translation stop codons in each reading frame and no protein products have been detected. Germline transcription is a required event for switch recombination. Deletion of the Iγ1 or Iγ2b exons and their promoters resulted in the inhibition of class switching to the corresponding genes (Jung *et al.* 1993; Zhang *et al.* 1993). Potentially, one function of germline transcription could be to maintain or participate in the initiation of locus accessibility, either by the act of transcription itself, or by the associated activity of transcription factors involved in chromatin accessibility such as the histone acetyltransferase p300/CBP (Ogryzko *et al.* 1996). Some evidence suggests that germline transcripts function in the mechanism of switch recombination. Conservation of the germline exon 3' splice donor sequence is required in addition to transcription for normal levels of switch recombination, suggesting that a correctly spliced germline transcript is required (Lorenz *et al.* 1995). Additionally, transcripts from the murine Sα region form a strong tri-molecular complex with the switch region *in vitro* (Reaban *et al.* 1994), suggesting they maintain accessibility to the recombination apparatus by disrupting the helix or by excluding nucleosomes from the switch region. The existence of *trans*-spliced germline transcripts also suggests a role of chimeric transcripts in the alignment of the two switch regions during recombination (Fujieda *et al.* 1996).

Germline transcription in all the murine downstream loci except γ1 are significantly reduced upon inactivation of the 3' LCR, suggesting that the LCR regulates germline transcription in addition to recombination. The presence of an enhancer immediately upstream of the GL-γ1 exon (Xu and Stavnezer 1992), suggests the independence of γ1 germline transcription from the 3' LCR could be through the activity of this enhancer.

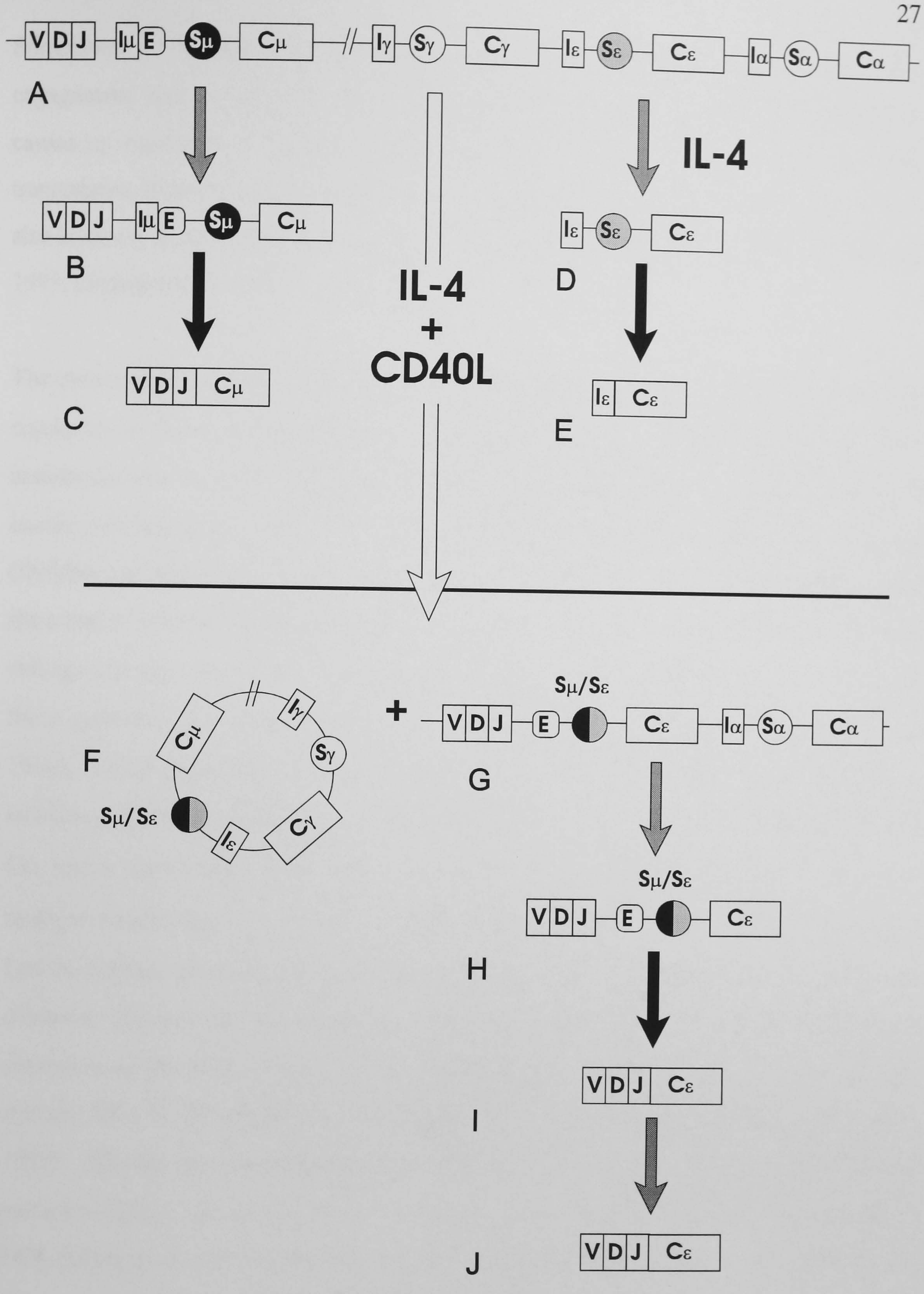


Figure 1.6. The mechanism of heavy chain recombination. (*A*) Prior to heavy chain switching, transcription occurs initiating at germline (I) exons in the heavy chain locus upstream of the mu switch region (Sμ), and after IL-4 stimulation, from Iε. Transcription proceeds through the respective constant region, producing a germline transcript (*B-E*). (*G*) In the presence of CD40 engagement on the B cell, recombination between Sμ and Sε can occur allowing production of a VDJ containing IgE transcript (*H, I*), and protein (*J*).

Stimulation of B cells through the cell contact mediated signal for switching, CD40 engagement, may work in part through the activation of the 3' LCR. CD40 engagement causes up regulation of 3' LCR activity (Grant *et al.* 1996). Consequently, many of the transcription factors known to be activated by the JNK cascade after CD40 engagement are also involved in the regulation of the LCR, including NF-kB, AP-1, and Elf-1, (Grant *et al.* 1995; Linderson *et al.* 1997).

The transcription factors NF-κB and BSAP and C/EBP have been found to participate in the regulation of many of the germline gene promoters and elements characterised in the immunoglobulin C_H locus, suggesting a co-ordinated regulation of the region. NF-κB is involved in regulation of the 3' LCR, and the germline gene promoter for murine epsilon (Delphin and Stavnezer 1995). C/EBP family members are involved in the regulation of the ε and γ1 germline genes (Lundgren *et al.* 1994; Delphin and Stavnezer 1995). The B cell-specific transcription factor (BSAP/PAX-5) is involved regulation of the 3' LCR and the ε germline gene (Singh and Birshtein 1993; Neurath *et al.* 1994; Michaelson *et al.* 1996). BSAP has also been shown to bind at multiple sites within the murine IgH locus, including the region 5' of Sγ2a, a segment downstream of Cα, at two sites upstream of Cα, and in Sμ (Waters *et al.* 1989; Liao *et al.* 1992; Xu *et al.* 1992). The existence of multiple binding sites for NF-κB, C/EBP, and BSAP raises the possibility that these factors mediate physical interactions between the LCR and the germline gene promoter elements. NF-κB has been shown to physically interact with Ets family members in the activation of the HIV enhancer in T cells (Bassuk *et al.* 1997) and with the Ets family member Elf-1 in the activation of the interleukin 2 receptor alpha-chain gene (John *et al.* 1995). NF-κB also physically interacts with the bZIP region of several C/EBP family members (Stein *et al.* 1993). The role of several Ets proteins in the regulation of the 3' LCR (Grant *et al.* 1995; Linderson *et al.* 1997), and of C/EBP factors in the regulation of the ε-germline gene (Delphin and Stavnezer 1995), suggests that NF-κB may also interact with these factors in the immunoglobulin locus. BSAP has been shown to physically interact with octamer binding proteins in its repression of the 3' LCR (Singh and Birshtein 1996), to block the binding of the Ets family member NF-αP during this repression, and to recruit Ets family members to the early B-cell-specific mb-1 promoter (Fitzsimmons *et al.* 1996). The potential of physical interactions between NF-κB, C/EBP, BSAP and Ets family members supports the idea that these factors aid in the communication

of the 3' LCR with the germline promoters by protein-protein interactions. A better understanding of the regulation of ε-germline transcription may allow the design of new anti-allergy strategies to control this important step in the mechanism of heavy chain switching to IgE.

1.10 The glucocorticoid potentiation of IgE secretion.

Glucocorticoids are used extensively to control the symptoms of allergy through their anti-inflammatory effects (for review see Schleimer 1996; Schwiebert *et al.* 1996). Endogenous glucocorticoids are secreted from the adrenal gland and attenuate both allergic inflammation as well as the general inflammatory response (Wilckens and De Rijk 1997). A variety of mechanisms of action are proposed to account for the activity of glucocorticoids on allergy. One mechanism suggests glucocorticoids inhibit inflammatory cell migration, potentially through an effect on the synthesis of cytokines that control the expression of adhesion molecules on endothelial cells, or through an affect on inflammatory cell activation. Glucocorticoids inhibit the secretion of the cytokines IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, RANTES, and GM-CSF from epithelial cells and peripheral blood mononuclear cells (Sousa *et al.* 1993; Kwon *et al.* 1994; Kwon *et al.* 1995; Brattsand and Linden 1996; Elenkov *et al.* 1996; Ghaffar *et al.* 1997). Suppression of the type 2 cytokines IL-4 and IL-13 is thought to reduce rhinitis in part through an inhibition of localised immunoglobulin isotype switching to IgE, as evidenced by a reduction in the expression of the germline gene transcript for IgE in the nasal epithelium (Durham *et al.* 1997).

Consistent with the effects on allergic symptoms, *in vivo* levels of allergen specific IgE decrease after steroid treatment (Crimi *et al.* 1990; Naclerio *et al.* 1993). In contrast, total IgE levels are unchanged or are moderately increase after treatment with corticosteroids (Gunnar *et al.* 1970; Henderson *et al.* 1973; Posey *et al.* 1978; Settipane *et al.* 1978; Klebl *et al.* 1994; Zieg *et al.* 1994). *In vitro*, the effect of glucocorticoids on total IgE is more pronounced. Glucocorticoids significantly increase IL-4 induced total IgE levels in cultures of peripheral blood mononuclear cells (Wu *et al.* 1991; Nusslein *et al.* 1994). Spontaneous IgE secretion is also increased by glucocorticoids in cultures of peripheral blood mononuclear cells (PBMC) in the absence of IL-4 (Kimata *et al.* 1995; Hiratsuka *et al.* 1996).

Little is known about the mechanism of the up regulation of IgE synthesis by glucocorticoids. The potentiation of IgE secretion from PBMC *in vitro* is monocyte dependent (Wu *et al.* 1991). Enhancement still occurs when glucocorticoid is added as late as day 6 after initiation of the culture, suggesting it still acts at the IL-4 independent stage of the IgE response. However, the potentiation of IgE synthesis in PBMC cultures may occur by a different mechanism than with purified B cell cultures. When glucocorticoid is added to purified B cells in the presence of IL-4 it replaces the cell contact-mediated signal for class switching, and unlike its potentiation of IgE synthesis with PBMC, it is not monocyte dependent and requires glucocorticoid at the onset of culture (Jabara *et al.* 1991). More work is needed to elucidate the mechanism of the enhancement of IgE synthesis in PBMC, as a model of the events that occur *in vivo*.

1.11 Conclusions.

Because of the central role of IgE and its high affinity receptor in allergy, a more clear understanding of the regulation of this ligand/receptor pair is needed to allow for the informed design of new anti-allergy strategies. A better understanding of heavy chain recombination could also help to elucidate how switch region sequence, and the regulation of transcription and chromatin accessibility are involved in the mechanism of recombination.

Chapter 3 describes the cloning and structure of the gene for the high affinity receptor for IgE. Sequence analysis of the upstream region of the gene for FcεRIα revealed a polymorphism that could be linked to allergy in the putative promoter region. In chapter 4 the heavy chain recombination junction of the IgE secreting myeloma U266 is analysed. The sequence of the recombination junction was found to reveal a prior switching event to IgA1. Recombination between the switch mu and switch alpha-1 regions occurred at an areas of homology between Sμ and Sα1. The germline gene transcript for IgE is cloned and characterised in chapter 5. The role of the B cell specific transcription factor BSAP/PAX-5 in the IL-4 and CD40 mediated up-regulation of ε-germline transcription is demonstrated in chapter 6. In chapter 7, the mechanism of the glucocorticoid potentiation of IgE synthesis is investigated. IgE enhancement was found to occur only after 10 days of glucocorticoid addition in *vitro*. Germline gene and productive epsilon transcription was not affected 4.5 days after glucocorticoid addition.

CHAPTER 2: Materials and Methods.

2.1 END LABELLING .

Kinasing: T4 polynucleotide kinasing was performed exactly as described by the Promega Biotec cycle sequencing protocol. 10 pMol oligonucleotide dried down to removed inhibitory NH_4^+ residue, and resuspended in dH_2O. 1 µL of T4 polynucleotide kinase buffer (Promega, Madison, WI), 3 µL 3000 ci/mMol (or 5 µL 5000 ci/mMol) γ^{32}P-ATP, ≤1 wk past activity date, 5 units of T4 polynucleotide kinase (Promega, 5u/µL), were added in a final volume 10 µL. The reaction was incubated at 37°C for 45 minutes, at 95°C for 5 minutes. The reaction was stored at -20°C. Typically, the kinased oligonucleotide was purified on 20% acrylamide gel (see 2.11 ACRYLAMIDE GEL ISOLATION); agarose leaves too much label contamination.

3' End Filling: First, 1 ρMol of 3' ends was prepared, devoid of free nucleotides. 5 µL of 10X Promega buffer D (150 mM NaCl), 2 µL 10 mM each dNTP except the nucleotide to be labelled, 5 µL α^{32}P-dNTP (3000 ci/mMol, 10 µci/µL, ≤2 weeks from activity date), 100 µg/mL BSA, dH_2O to a total volume of 49.5 µL, and 0.4 µL Klenow polymerase (New England Biolabs, Beverly MA) were added to the DNA. The reaction was incubated at room temperature for 30 minutes, 2 µL 10 mM unlabelled dNTP (same nucleotide as the labelled one) was added to chase, with 0.4 µL Klenow polymerase, and incubated at room temperature for 30 minutes. If needed, the DNA was purified on an acrylamide gel (see 2.11 ACRYLAMIDE GEL ISOLATION).

2.2 T4 BLUNT ENDING

1 ρMol of 5' or 3' overhang ends was mixed with 5 µL 10X polymerase buffer (or any restriction enzyme buffer), 1 µL 10 mM dNTPs, 100 µg/mL BSA, 2 units T4 polymerase (New England Biolabs, Beverly, MA), and dH_2O to a total volume of 50 µL. The reaction was incubated at 12°C for 60 minutes, phenol extracted, and precipitated.

2.3 PLASMID PREPARATION

For restriction analysis, the Birnboim plasmid DNA mini-preparation procedure was used [Birnboim, 1983 #477]. For DNA sequencing the Promega (Madison, WI) mini-preparation kit was used, exactly as described in the manufacturer's protocol except that RNase digestion containing 50 µg/mL RNase A was performed at the neutralisation step.

Birnboim Plasmid Mini-Prep Method (adapted from Birnboim 1983): *E. coli* bacteria were inoculated into 1.75 mL (or for 15 mL, in parentheses) Terrific Broth (Sambrook *et al.* 1989) with 50 µg/mL ampicillin, and grown to full density or overnight. The culture was spun in an Eppendorf tube (Falcon 2057 or 14 mL, Becton Dickinson, San Jose, CA.) to produce a cell pellet. The pellet was resuspended in 200 µL (1.6 mL) of Solution I (50 mM glucose, 25 mM Tris pH 8, 10 mM EDTA). 400 µL (3.2mL) of fresh Solution II (0.2 N NaOH, 1% sodium dodecyl sulphate: NaOH added first and mixed) was added. 300 µL (2.4mL) of Solution III (14.7 g of KCl, 5.75 mL of glacial acetic acid, total volume of 50 mL) was added. The tube was spun for 5 minutes in a micro-centrifuge at 4°C, and the supernatant was retained. The supernatant was phenol extracted one time with 2 volumes phenol, one time with phenol/$CHCl_3$ (1:1), and once with $CHCl_3$. One volume of ethanol was added, the precipitated was spun in a micro-centrifuge for 5 minutes, and the supernatant aspirated. The pellet was washed with cold 70% ethanol and dried in a Speedvac. The pellet was resuspended in 100 µL 10mM Tris-HCl pH 8, 0.1 mM EDTA (TE) with 50µg/mL RNase A and stored at -20 C. If an RNA free preparation was required, the supernatant from step 6 was precipitated with ethanol, washed with 70% ethanol and resuspended in TE, and 50 µg/mL RNase A was added. After 1-2 hour at 37°C the DNA was phenol extracted, chloroform extracted and lastly spun through a Sephadex G-50 column equilibrated in TE.

2.4 PREPARATION OF ssDNA FROM PHAGEMID (*eg* BLUESCRIPT) CLONES.

A. Preparation of helper phage stock:

Materials: Top agar consists of L broth with 0.7% agar (Sambrook *et al.* 1989). *E. coli* BB4 was obtained from Stratagene (La Jolla, CA). Tetracycline 12.5 mg/mL (1000x) was dissolved in 50% ethanol. L plates consist of L broth with 1.5% agar. The helper phage stock was titred as described below every one or two months.

Method: The rec^+ f^+ bacteria strain BB4 was plated on selective media (tetracycline, 12.5 μg/mL) to ensure the presence of the f-factor. The bacteria was grown L broth (with tetracycline) overnight or to confluence. Top agar was melted and cooled to 42°C. A dilution series of helper phage was prepared in L broth assuming the phage stock was 10^{10} plaque forming units (PFU) per mL or less. 100 μL of bacteria was combined with a dilution of helper phage and 3 mL of top agar, and quickly poured on a L plate warmed to room temperature. The plate was incubated overnight at 37°C. 3 mL of L broth was added to a plate that was confluent (no plaques visible) and was agitated on a shaker at room temperature for 1 hour. The L broth was collected with a pipette and the cells were spun out at 10,000 RPM for 10 minutes. The supernatant was incubated at 65°C for 10 minutes to kill the remaining bacteria. The phage stock was titred as described above and stored at 4°C. The titre was normally about 10^{10} PFU/mL.

B. Infection of bacteria with helper phage:

Materials: Ampicillin 50 mg/mL (1000x) in dH_2O was filter sterilised. Kanamycin 100 mg/mL (1000x) in dH_2O was filter sterilised. Tetracycline 12.5 mg/mL (1000x) was dissolved in 50% ethanol. SOB or 2X-YT medium were prepared as described in Sambrook *et al* (1989). Helper phage M13K07 was obtained from Promega (Madison, WI) or VCSM13 was obtained from Stratagene (La Jolla, CA).

Method: The phagemid was introduced into a rec^+ f^+ bacteria by transformation or by mating the f-factor from BB4 into a rec^+ strain containing the phagemid. *E. coli* BB4 was the simplest to use, as the f-factor can be selected for by growing in the presence of tetracycline.

Mating protocol:. a) To mobilise the f-factor and phagemid into the same bacterial cell, both the strain containing the phagemid, and BB4 were grown and to confluence. BB4 was grown in the presence of 12.5 mg/mL tetracycline, and the phagemid containing strain with 50 μg/mL ampicillin. b) The residual antibiotics were washed from the bacteria by centrifuging the cells in a micro-centrifuge and resuspending them in a few mL of L broth. A drop of each culture was combined together on a L plate (no antibiotics) and allowed to mate the bacteria for 4-6 hours at 37°C. c) The bacteria were inoculated with a bacterial loop on an L plate containing tetracycline and ampicillin to select for the f-factor and the phagemid, respectively. The plate was incubated less than 18 hours at 37°C. The efficiency of mating can be as great as 1 in 10.

From a plate containing a selection for both the phagemid and the f-factor, an overnight liquid culture was grown containing the antibiotics ampicillin and tetracycline. The antibiotics were washed from the medium, 200 μL of the culture was inoculated into 5 mL SOB or 2X-YT media without antibiotics, 150 μL of helper phage (5×10^9 PFU/mL or better) was added, and the culture was incubated with agitation for 1 hour at 37°C. After 1 hour 100 μg/mL of kanamycin was added to select for infection by the helper phage, and the culture was incubated for a further 4-6 hours.

C. Extraction of ssDNA from phage:

Materials: 40% Polyethylene glycol (PEG) 8000, 5 M sodium acetate pH 7, saturated ammonium acetate (≈10 M).

Method: The cells from above were centrifuged at 10,000 RPM for 10 minutes. To the supernatant 1/9 of the volume of 5 M NaOAc pH 7 and 1/9 of the volume of 40% PEG 8000 were added and mixed thoroughly. The phage were precipitated for 15 minutes at

room temperature. The precipitate was centrifuged at 10,000 RPM for 10 minutes and the supernatant was withdrawn. The pellet was centrifuged again for 10 minutes and the remaining supernatant was removed . The pellet was usually barely visible. The pellet was resuspended in 200 µL of 10mM tris-HCl pH 8, 0.1 mM EDTA (TE) by freeze/thaw cycles if necessary, but vortexing was avoided. The DNA was extracted with two volumes of phenol once, and two volumes of phenol/$CHCl_3$/Isoamyl alcohol 25:25:1 until only a small amount of debris remained at the interface and 0.25 volumes of 10 M ammonium acetate and 2.5 volumes of ethanol were added. The DNA was precipitated for 10 minutes on ice, spun in a micro-centrifuge for 30 minutes at 4°C, and the pellet was washed in 70% ethanol, dried and resuspended in 20 µL dH_2O. The DNA was stored at -20°C. The concentration was estimated using 1 µL of the DNA against 0.5 µg of ssDNA of known concentration on an agarose gel.

2.5 GITC/PHENOL METHOD FOR THE EXTRACTION OF RNA. (modified from Chomczynski and Sacchi 1987)

Solutions: Solution D: 4 M guanidine isothiocyanate (GITC), 25 mM sodium citrate, pH 7, 0.5% sodium lauryl sarcosinate, 0.1% Antifoam A (added fresh, 1 µL per 1 mL solution D), 0.7% 2-mercaptoethanol (added fresh, 7 µL per 1 mL solution D). To make solution D, GITC was dissolved at 50°C and sodium citrate and sodium lauryl sarcosinate were added . This solution was stored at room temperature for months. Antifoam A and 2-mercaptoethanol were added on the day of the extraction. Milli-Q dH_2O was used as a source of RNase-free water, as diethyl pyrocarbonate (DEPC) treatment (Sambrook *et al.* 1989) reduces the template activity for enzymes. 10 M ammonium acetate ($(NH_4)_2OAc$) was made with RNase-free water. Phenol (AquaPhenol, Appligene, Illkirch, Fr) was equilibrated by mixing with an equal volume of water. 5:1 Aquaphenol:$CHCl_3$/IAA was centrifuged to removed water and made fresh). 70% ethanol was made with RNase-free water.

Materials: The following plastics were used without DEPC treatment: Prepackaged pipette tips (preferably filter tips), sterile screw-cap Eppendorf tubes (Scotlab, Coatbridge, Scotland), or others, except Sarstedt which break), and sterile disposable plastic tubes.

Method: (A) For 10^7 cells: First, the cells were pelleted and supernatant was withdrawn. The cells were not washed and were frozen at -70°C as a pellet. 0.4 mL of solution D (but no less than 4 volumes of the cell pellet with residual medium) was added to the cell pellet with a 1 mL syringe and a 26 gauge needle. The cells were immediately broken up by forcing the lysis solution and cells through the syringe repeatedly until the lysate was homogeneous. More solution D was added if the pellet was too viscous. Homogenization was continued an additional 10 times to shear the DNA. The lysate was transferred to an Eppendorf tube with 2 volumes of Aquaphenol/$CHCl_3$/IAA (5:1), mixed and cooled on ice for 15 minutes. The extraction was spun for 20 minutes at 4°C in a micro-centrifuge or at 10,000 RPM in a Sorvall. The aqueous phase was retained. If the interface was a large volume of the aqueous layer, it was back extracted by adding half the original volume of solution D to the remaining phenol/$CHCl_3$ and repeating step 3. The aqueous phases were pooled from both extractions and extracted again with phenol/$CHCl_3$. The phenol/$CHCl_3$ extractions were repeated until only a fine layer of debris was left at the interface of the phases. The lysate was extracted once with $CHCl_3$/Iso amyl alcohol 25:1. 0.5 volumes of ethanol was added and the RNA was precipitated overnight at -20°C, or 30 minutes on ice if in a hurry. The precipitate was spun at 4°C for 30 minutes in a micro-centrifuge, or 10,000 RPM in a Sorvall, washed in ice cold 70% ethanol, and micro-centrifuged at 4°C for 10 minutes. The RNA was resuspended in 100 µL dH_2O, if necessary by repeated cycles of freeze/thaw/vortex. The RNA was kept on ice when thawed. 0.25 volumes of $(NH_4)_2OAc$ was added to the RNA and mixed, 2.5 volumes ethanol was added , snap-frozen on liquid N_2, and left on ice for 10 minutes. The precipitate was micro-centrifuged at 4°C for 30 minutes . The pellet was washed with ice cold 70% ethanol, micro-centrifuged for 10 minutes and then vacuum dried. The RNA was resuspended in dH_2O (about 100 µL for 10^7 cells), and stored at -70 C. RNA concentration was estimated against a control of known concentration, by comparing the intensity of ribosomal bands after agarose gel electrophoresis in 1x tris borate gel running buffer (TBE) in the presence of ethidium bromide. If no degradation occurred, the 28S ribosomal band was 2-3x the intensity of the 18S. If the 28S/18S ratio was less, then degradation occurred. The concentration of the control was determined by A_{260} as described in Sambrook *et al* (1989).

(B) For 1 cell to $5x10^6$ cells: The same method as described above was used except that *E. coli* Ribosomal RNA (#206 938, Boehringer Mannheim, Mannheim Germany) was added at 100 µg/mL and at least 0.1 mL of solution D was used. The concentration was estimated by probing for a control transcript such as β-actin and comparing this signal against that of an RNA preparation of known concentration. The RNA was resuspended in 20-100 µL of dH_2O.

2.6 RNA EXTRACTION: CsCl/Tabletop Ultracentrifuge.

Method: Less than $5X10^8$ cells were centrifuged at 1,200 RPM for 10 minutes, and not washed. 2.2 mL GITC lysis buffer (4 M GITC, 0.1 M Tris pH 7.5, 1% β-mercaptoethanol, added just before use) was added to the cell pellet with a syringe containing a 25-27 gauge needle. The cells were homogenised quickly until the lysis was complete and continued for another 10 times. Sodium lauryl sarcosinate was added to a concentration of 0.5% (using a 10% solution). With a cut off P1000 tip, the lysate was carefully layered onto 0.83 mL of CsCl solution (5.7 M CsCl, 10 mM EDTA pH 8) in a Beckman Polyallomer tube (no. 349623 Beckmann Instruments, Fullerton, CA). The gradient was centrifuged at 70,000 RPM for 3 hours in a TL100.3 rotor (Beckmann Instruments, Fullerton, CA). The lysate was aspirated to just below the top of the CsCl cushion. With a hot scalpel, or razor blade, the top was cut off of the tube just above the liquid level. The remaining solution was removed and washed with 80% ethanol. 100 µL phenol/chloroform and 100 µL dH_2O were added and the pellet was transferred to an Eppendorf tube. The RNA pellet was resuspended completely by repeated cycles of freezing, thawing, and vortexing. 0.25 volumes of saturated (10 M) NH_4OAc was added and the RNA was spun in a micro-centrifuge. To the supernatant was added 2.5 volumes of ethanol. The RNA was cooled in liquid N_2, precipitated 10 minutes on ice, and spun for 30 minutes in a refrigerated micro-centrifuge. The pellet was washed with cold 80% ethanol, and resuspended in 50-400 µL dH_2O by freeze/thaw/vortexing. 0.25 volumes NH_4OAc and 2.5 volumes ethanol, were added to the RNA, which was then cooled on liquid N_2, micro-centrifuged, and washed. The tube was spun again for 1-2 minutes and the last traces of ethanol were removed with a pipette tip. The pellet was Speedvac dried, resuspended it in dH_2O, and stored it at -80°C.

2.7 SEMI-QUANTITATIVE RT-PCR.

Materials: cDNA synthesis: Pre-packaged plastic tips and tubes, unopened chemicals and Milli-Q water were assumed to be RNase free, therefore diethyl pyrocarbonate (DEPC) treatment was not performed.

Materials PCR: Filter pipette tips were used if possible to avoid cross contamination.

cDNA synthesis: Less than 5 µg of RNA was brought to a volume of 10.2 µL with dH_2O. The RNA was kept on ice for only a few minutes before cDNA synthesis, to avoid degradation. The RNA was heated to 70^0C for 2 minutes and cooled on ice for 1 minute. 8.1 µL cDNA mix was added; per reaction: 4 µL 5X cDNA buffer (Gibco BRL/Amersham Life Sciences, Arlington Heights, IL), 1.6 µL 10 mM dNTPs, 0.5 µL 2 µg/µL random 10 base oligonucleotides (10 mers), 2 µL 100 mM dithiothreitol (Gibco/BRL), 0.7 µL RNase inhibitor, 1 µL murine-Moloney leukaemia virus reverse transcriptase (M-MLV RT, Gibco/BRL #28025-013) and incubated 37^0C 60 minutes

Oligonucleotide kinasing (as Promega cycle sequencing protocol): 10 pMol oligonucleotide was dried down to removed inhibitory NH_4^+ residue and resuspended in dH_2O. 1 µL buffer (Promega, Madison, WI), 3 µL 3000 ci/mMol (or 5 µL 5000 ci/mMol) γ^{32}P-ATP, 5 units T4 polynucleotide kinase (5 u/µL, Promega, Madison, WI) was added and the reaction was brought to a final volume of 10 µL with dH_2O. The reaction was incubated at 37°C for 45 minutes, and then 95°C for 5 minutes to denature the kinase.

Polymerase chain reaction (PCR): A master mix was made of (per reaction) 14.05 µL dH_2O, 2 µL 10X Buffer (including 15 mM $MgCl_2$, Promega), 0.4 µL 10 mM dNTPs, 0.2 µL 100 µM oligonucleotide A, 0.2 µL 100 µM oligonucleotide B, 0.4 µL (or less) kinased oligonucleotide A or B, 0.1 µL polyoxyethylenesorbitan monolaurate (tween 20), mixed well, and 0.05 µL Taq polymerase (Promega 4.5 units/µL) was added. 18 µL of the master mixture was added per reaction and 2 µL (or less) cDNA. 3 drops (from a P-

1000) of mineral oil was added to each tube prior to PCR. After the PCR part of the reaction was run on a 4% polyacrylamide gel in 1X Tris borate gel running buffer (TBE) and dried the gel before exposing.

Quantitation: Quantitation was performed by a phosphor-imaging, using a GS525 phosphoimager (Biorad, Hercules, CA.), and integrating the digitised bands with Molecular analyst software.

2.8 PRIMER EXTENSION.

Materials: Milli-Q water, and pre-sterilised disposable plastics were assumed to be RNase free. Diethyl pyrocarbonate (DEPC) was not used, as it degrades template activity significantly. 10-50 µg CsCl isolated total RNA was used and checked on Northern gel for integrity.

Method: An oligonucleotide kinased with >5000 ci/mMol γ^{32}P-ATP was purified on a Superfine Sephadex G-25 superfine column, made with a silanised Pasteur pipette. 50-100 µL fractions were taken, because the oligonucleotide and the unincorporated peaks travel very close together. For the annealing reaction, 1.5 µL 10X Annealing Buffer (1.5 M KCl, 0.1 M Tris pH 8.3, 10 mM EDTA), 1 µL Oligonucleotide (less than 1 pMol, but the quantity needs to be adjusted for each reaction), 12.5 µL RNA were added, and the reaction was incubated for 10 minutes at 65°C, and 1.5 hour at the annealing temperature which was calculated as for PCR (T_a = 16.6Log[NaCl or KCl] + 0.41(%GC) + 81.5 - 675/length + 10°C). The reaction was cooled slowly (30-60 minutes) to 37°C. 27.7 µL of extension mixture (per reaction) containing 16 µL dH_2O, 0.125 µL 1 M Tris pH 8 (at room temperature), 0.625 µL, 1 M Tris pH 8.3 (at 42°C) 0.45 µL, 1 M $MgCl_2$, 0.45 µL 5 mg/mL actinomycin D, 50 µg/mL (in ethanol, Calbiochem La Jolla, CA), 2.25 µL each 100 mM dNTP (in dH_2O, Pharmacia), 0.563 µL 2 M KCl and 0.45 µL 100 mM DTT were added. 2.3 µL M-MLV reverse transcriptase (Gibco BRL/Amersham Life Sciences, Arlington Heights, IL 28025-013) was added, and the reaction was incubated at 37°C for 1 hour. 6 µL 0.5 M EDTA, 100

μL TE and 1 μL 10 mg/mL Boiled RNase A was added, and the reaction was incubated at 37°C for 30 minutes. 38 μL saturated NH_4OAc (≈10 M) was added, the reaction was phenol/$CHCl_3$ extracted, and transferred to a silanised tube. 530 μL of ethanol was added and the precipitate was cooled on liquid N_2, and the DNA was incubated on ice at least 15 minutes. The precipitate was micro-centrifuged at 4°C for 30 minutes, washed with 80% ethanol, and all the supernatant was withdrawn. The pellet was dried to completion in a Speedvac. 4-5 μL of formamide loading buffer was added to the pellet, it was heated at 95°C for 3 minutes, and run on a sequencing gel against a sequencing ladder as a marker, priming with the same end-labelled oligonucleotide.

2.9 S1 NUCLEASE PROTECTION (Chapter 5).

An S1 probe was generated as follows: 2.5 μM of oligonucleotide Q CCATTTGGTGCCTGTGGATGGTGTG (227-251 of the human germline epsilon gene), was dried and resuspended it in 6.75 μL dH_2O, 1.25 μL 10X T4 polynucleotide kinase buffer (Promega Biotec, Madison, WI), 4 μL 3000 ci/mMol ^{23}P-γATP, 5 units of T4 polynucleotide kinase, incubated at 37°C for 1 hour, and 95°C for 5 minutes. Single stranded DNA from a 2.75 kb Kpn I-Hind III clone of the germline epsilon locus cloned into Bluescript was made by the "ssDNA preparation procedure" above. The "Sequenase" annealing procedure was followed to anneal the oligonucleotide to the template: 12 μL of 100 ng/mL of the 2.75 kb Kpn I-Hind III clone was added to 4 μL of the kinased oligonucleotide Q and 4 μL 5X "Sequenase" buffer (Amersham Life Sciences-USB, Arlington Heights, IL), heated to 70°C for 2 minutes, and annealed by cooling to 35°C for 30 minutes. 0.4 μL of a 10 mM mixture of dATP, dCTP, dGTP, and dTTP was added to the annealing reaction along with 8 μL dH_2O, 1 μL 0.1 M dithiothreitol, and 0.6 μL Sequenase T7 polymerase, and incubated at 37°C for 15 minutes. 1 μL of BSA, 144 μL of dH_2O, 20 μL of 10X Pst I buffer, and 40 units of Pst I was added to the reaction, and it was incubated for 1 hour at 37°C. The reaction was phenol extracted, and precipitated with 500 μL ethanol. The single stranded probe was isolated on a 4% polyacrylamide/urea sequencing gel.

5 μL (10 μg) of total RNA in from PBMC induced for 4 days with IL-4 was added to 5 μL hybridisation buffer containing 20% polyethylene glycol 8000 80 mM piperazine-1,4-bis-(2-ethanesulfonic acid), 800 mM NaCl, 2 mM EDTA, and 1/80th of the probe preparation. The samples were boiled for 10 minutes, and incubated at 70°C for 30 minutes. 300 μL of warm S1 digestion buffer was added, containing 280 mM NaCl, 30 mM NaOAc pH 4.8, 4.5 mM $ZnSO_4$, 20 μg/mL boiled calf thymus DNA, and 200 units S1 nuclease (Boehringer Mannheim, Mannheim Germany). The reaction was incubated at 45°C for 30 minutes. 75 μL of termination buffer was added, containing 2.5 M NH_4OAc, 50 mM EDTA, and 170 μg/mL yeast tRNA (Boehringer Mannheim, Mannheim Germany). Two volumes of ethanol were added, the tube was cooled on liquid N_2, and incubated on ice for 10 minutes. The precipitate was spun in a refrigerated micro-centrifuge for 90 minutes, washed 2 times in 80% ethanol, and dried completely in a Speedvac. The pellet was resuspended in 4 μL formamide loading buffer, denatured at 95°C for 3 minutes, and loaded onto a 6% acrylamide/Urea sequencing gel. A sequencing ladder was used as a marker, with the same 3' end labelled oligonucleotide Q for the sequencing primer as for synthesis of the S1 analysis probe.

2.10 BACTERIAL COLONY PCR

Colony isolation: A visible amount (0.1-1 μL) of a bacterial colony was picked from a plate with a Pipetteman. The colony was often several days old. The bacteria were added to 100 μL of TE in a micro-centrifuge tube. Often, 10-20 colonies were pooled into one tube. The suspension was vortexed, boiled for 5 minutes, cooled, micro-centrifuged briefly, vortexed again and micro-centrifuged for 30 sec in a micro-centrifuge to pellet the debris.

PCR: 2 µL bacterial lysate was added to 18 µL of PCR mixture, composed of (per reaction) 15.05 µL dH_2O, 2 µL 10X PCR buffer (containing 1.5 mM $MgCl_2$, Promega, Madison, WI), 0.4 µL 10 mM dNTPs (mixture of all four), 0.2 µL 100 µM oligonucleotide A, 0.2 µL 100 µM oligonucleotide B, 0.1 µL Tween 20, and 0.05 µL Taq polymerase (Promega 4.5 U/µL). 3 drops (from a P-1000) of heavy paraffin oil was added to each tube. The PCR was cycled 30-40 times at 94°C for 1 minute, at the annealing temperature for 1 minute, and at 74°C for 1 minute. The annealing temperature was calculated as follows: (T_a = 16.6Log[NaCl or KCl] + 0.41(%GC) + 81.5 - 675/length + 10°C).

2.11 ACRYLAMIDE GEL ISOLATION.

A sample of DNA was on a non-denaturing acrylamide gel, using either 4 µg unlabelled or 0.5 µL end labelled low molecular weight marker (MspI Bluescript SK^+) in 0.5X TBE (Sambrook *et al.* 1989). If the DNA was unlabelled, it was soaked in 1 µg/mL ethidium bromide for 20 minutes and the band was cut out on a UV box. If labelled, the gel was exposed a short time with autoradiography film. The bands were cut out , transferred to a screw-top Eppendorf tube, and macerated with a P1000 tip. The gel fragments were washed off the tip with 100-200 µL of TE, and the volume was adjusted to ≤ 200 µL with TE, 100 µL saturated NH_4OAc was added, and the tube was put on roller at 37°C for 2 hour (or 4°C overnight). The gel debris was micro-centrifuged, the supernatant was transferred to a 1.5 mL Spin-x sterilisation filter (Cat#8160 Costar, Cambridge, MA), micro-centrifuged, and saved on ice. 200 µL of TE was added to the pellet, which was resuspended and put on a roller at 37°C for 2 hours. The gel fragments and buffer were transferred to the top of the previous Spin-x filter and spun again. The filtrate was transferred to a screw-top Eppendorf, 2 volumes ethanol was added , the precipitate was frozen on liquid N_2, and incubated on ice for 10 minutes. The DNA was micro-centrifuged for 90 minutes at 4°C, washed with cold 80% ethanol, micro-centrifuged for 20 minutes, and completely dried in a Speedvac.

2.12 AGAROSE GEL ISOLATION.

Typically, a low melting point agarose (Gibco/Amersham Life Sciences, Arlington Heights, IL) gel was run in 1X TBE, 1 μg/mL ethidium bromide (Sambrook *et al.* 1989) at a low voltage (in cold room if more than 1-2 hours). The band of interest was cut out of the gel and transferred to a screw-top Eppendorf tube. 100 μL of saturated NH_4OAc was added, the isolate was diluted to 0.5% agarose with TE, heated at 70°C for 5 minutes, 2 volumes phenol were added, and the tube was micro-centrifuged for 5-10 minutes at room temperature. The supernatant was transferred to a new tube, 1 volume phenol was added, and the extractions were repeated until no debris remained. 2 volumes of phenol:$CHCl_3$ isoamyl alcohol was added to the supernatant and the tube was micro-centrifuged. If necessary, the supernatant was concentrated by extracting it with 2 volumes of 2-butanol serially until the volume was ≤500 μL. The supernatant was extracted with 2 volumes $CHCl_3$:isoamyl alcohol (1:24). 2 volumes of ethanol were added to the supernatant, which was frozen it on liquid N_2, and micro-centrifuged for 90 minutes at 4°C. The pellet was washed with cold 80% ethanol, micro-centrifuged for 20 minutes, and completely dried in the Speedvac.

2.13 BACTERIAL ELECTROPORATION.

The bacterial electroporation method was followed exactly as described in the "Gene Pulser" (Biorad, Hercules, CA.) protocol, using 0.2 mL cuvettes, and 50-100 μL cells either prepared in dH_2O instead of buffered solution, or purchased from Stratagene (La Jolla CA).

After electroporation, 1 mL of L broth (Sambrook *et al.* 1989) was added and the cells were shaken for 45 minutes. At least 200 μL of the cells were mixed on a 50 μg/mL ampicillin L plate with 50 μL of a 2% solution of 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) and 2 μL of 1 mg/mL isopropylthio-β-galactoside (IPTG), left to dry uncovered until liquid was adsorbed, then incubated inverted at 37°C overnight.

2.14 LAMBDA LIBRARY SCREENING (Chapter 3).

The FcεRIα gene was screened using a λ EMBL-3 library (#HL1006d, Clonetech, Palo Alto, CA). The bacterial strain LE392 was used for screening the library. Approximately $5X10^4$ phage were added to an overnight culture of LE392, incubated at 37°C for 20 minutes, and added to 3 mL 10^6 plaques were screened on 20 140 mm plates. Inserts were excised from vector with SalI.

2.15 DNA FRAGMENT SIZE ESTIMATION (Chapter 3)

After the DNA to be sized was run on an agarose gel alongside molecular weight markers, a standard curve was made by plotting the distance of the migration of the markers from the origin (well), on a semi-log graph, and fitting a straight line through them. After the distance of the migration of each unknown band was measured, the apparent molecular weight was read from the standard curve .

2.16 GERMLINE TRANSCRIPT CLONING (Chapter 5).

cDNA Synthesis: RNA was isolated as described in: 2.5 GITC/PHENOL METHOD FOR THE EXTRACTION OF RNA, from 10^7 PBMC induced for 5 days with IL-4. First and second strand cDNA was isolated with the Pharmacia cDNA synthesis kit (Amersham Life Sciences, Arlington Heights, IL) from 0.5 μg of PBMC total RNA and 5 μg of yeast tRNA (Boehringer Mannheim, Mannheim, Germany). The resulting S-300 column effluent cDNA was treated with 3 units of T4 polymerase, heat inactivated at 70°C for 5 minutes, and digested with 20 units of HincII for 5 hours. The digested cDNA was phenol extracted and run over a S-300 column again for 2 minutes at 1500 RPM. To a total volume of 50 μL of column effluent 2.5 μL of 10 mM ATP and 1000 units of high concentration T4 ligase (New England Biolabs, Beverly MA) was added, and the reaction was incubated at 22°C for 10 hours. The recircularised cDNA was phenol extracted, ethanol precipitated, and resuspended 17 μL of dH_2O.

ε Germline Transcript 5' End Cloning: 5 μL of the recircularised cDNA was added to a PCR reaction exactly as described in the "semi-quantitative RT-PCR" method above, with oligonucleotide H and oligonucleotide F (Table 5.1) at 94°C 0.5 minutes, 65°C 1 minutes, 74°C 1 minutes for 40 cycles. The PCR reaction was digested with Ava I and Xba I and agarose gel isolated. 50% of the isolated PCR product was added with 20 ng of Ava I/Xba I digested (agarose gel isolated) Bluescript SK^+ (Stratagene, La Jolla, CA), and ligated with 200 units of T4 ligase in a volume of 10 μL at 12°C overnight. *E. coli* were transformed by the "electroporation" method above with 30% of the ligation mixture. Transformants were screened by the "bacterial colony PCR" method above, and screened with oligonucleotide B (Table 5.1) and the M13 reverse oligonucleotide (New England Biolabs, Beverly MA). Positive transformants were sequenced by the "Sanger" method below.

ε Germline Transcript 3' End Cloning: 5 μL of the recircularised cDNA was added to a PCR reaction exactly as described in the "semi-quantitative RT-PCR" method above, with oligonucleotide G and oligonucleotide Cε4 (Table 5.1) at 94°C 0.5 minutes, 65°C 1 minutes, 74°C 1 minutes for 40 cycles. The PCR reaction was treated with T4 polymerase as described in: 2.2 T4 BLUNT ENDING above, and agarose gel isolated. 50% of the isolated PCR product was added with 20 ng of Eco RV digested (agarose gel isolated) Bluescript SK^+ (Stratagene, La Jolla, CA), and ligated with 200 units of T4 ligase in a volume of 10 μL at 20°C for 6 hours. *E. coli* were transformed by the "electroporation" method above with 30% of the ligation. Transformants were screened by the "bacterial colony PCR" method above, and screened with oligonucleotides Cε4 and either the M13 reverse, or -20 oligonucleotides (New England Biolabs, Beverly MA). Positive transformants were sequenced by the "Sanger" method below.

2.17 SEQUENCING.

Sanger *et al* (1977) Sequencing . The "Sanger" sequencing method was followed exactly as described in the "Sequenase" protocol (Amersham Life Sciences-USB, Arlington Heights, IL), except for the denaturation step for dsDNA. Denaturation was performed with 3 μg plasmid DNA, brought to a 16 μL total volume with dH_2O. 4 μL

of 1 M NaOH/1 mM EDTA was added to the DNA and mixed quickly. The reaction was incubated at 37°C for 10 minutes. 5 µL of saturated NH_4OAc was added to the denatured DNA, with 63 µL ethanol, and it was frozen on liquid N_2, and incubated the reaction on ice for 10 minutes. The tube was micro-centrifuged for 30 minutes at 4 °C, washed with cold 80% ethanol, micro-centrifuged for 30 minutes at 4 °C, and completely dried in the Speedvac.

Cycle Sequencing. The cycle sequencing protocol (Promega, Madison WI) was used exactly as described in the manufacturers' recommendation.

Notes: Sequencing of the U266 splice junction (Chapter 4) was performed on ssDNA (see 2.4 PREPARATION OF ssDNA FROM PHAGEMID CLONES). Sequencing of the FcεRIα chain gene (Chapter 3) was performed directly on lambda phage DNA using the cycle sequencing protocol according to the protocol above.

2.18 GERMLINE ε PROMOTER REPORTER CONSTRUCTS:

In order to obtain the human ε germline (GL-ε) promoter construct, a 423 bp fragment of the promoter, that ends 9 bp upstream of the splice site for Cε, was inserted into the promoterless pGL3 basic vector (Promega, Madison, WI), that contains a luciferase (luc) reporter gene. The fragment was obtained by PCR amplification of genomic DNA from non-atopic human lymphocytes with Pfu DNA polymerase (Stratagene, La Jolla, CA) as follows: A forward primer 5'-CCTGGGAGTGAGTACAAGGTGAG, and as reverse primer 5'-GGTGGGCTGGGATACCTGAAG (nucleotide 25-47 and 667-687 of Genbank accession no. X56797) were added to 10 µg genomic DNA, 5% dimethyl sulfoxide, 6 units of PFU polymerase (exactly as described in manufacturers protocol) in a 100 µL total volume. The reaction was cycled 40 times at 95°C for 1 minute, 64°C for 1 minute, 72°C for 1 minute and agarose gel isolated. The PCR product was digested with Rsa I and Hae II, the 3' end was made blunt (see 2.2 T4 BLUNT ENDING), agarose gel purified, and ligated into the SmaI site of pGL3, thus obtaining the ε–GL promoter construct. The insert and the junctions were sequenced by Sanger sequencing (Sanger *et al.* 1977) using the Sequenase version 2.0 kit (protocol (Amersham Life Sciences-USB Arlington Heights, IL).

The deletion mutant lacking the BSAP binding site (BSAP del) was generated by PCR assembly of two fragments, using the ε GL promoter construct as the template for amplification. A 274 bp PCR product (BSAP Del 1) was obtained using as forward primer 5'-CACCCCATTTTTAGCTCCCAGGCTCCACTGC, that introduces a 27 bp deletion, and corresponds to nucleotide 94-151 in the human germline epsilon gene. The reverse primer was 5'-GCAGTTGCTCTCCAGCGGTTC (GL4 primer, Promega), that maps to position 146-165 in pGL3. Another PCR product of 357 bp (BSAP Del 2) was generated using as forward primer 5'-CTAGCAAATAGGCTGTCCC (RVprimer3, Promega), that maps to position 4761-4780 in pGL3, and as reverse primer 5'-AGCTAAAAATGGGGTG that maps to position 94-109 in the human germline epsilon gene. The two PCR products were gel purified and combined into a PCR reaction using primers RVprimer3 and GL4, then digested with Kpn I and Hind III to generate a 470 bp fragment, and directionally cloned into Kpn I/Hind III digested pGL3. The BSAP mut1 construct was generated using as a forward primer RVprimer3, and as a reverse primer 5'-GGGAGCTCTTCCTCAG (nucleotide 117-132 in the human germline epsilon gene), where a G was replaced by a T as underlined in the sequence. The PCR product was digested with Kpn I and SacI to generate a 312 bp fragment, and inserted into the 4923 bp Kpn I/Sac I fragment of the GLε promoter construct. The deletion and the mutation were confirmed by "Sanger sequence analysis". All plasmids were prepared for transfection by 2 rounds of purification on CsCl density gradients.

2.19 CELL CULTURE, CELL LINES.

The B cell lines BL-2 (Gauchat *et al.* 1992), BJAB (Clements *et al.* 1975), and Namalwa (Middleton *et al.* 1991), were cultured in Yssel's medium (without insulin and transferrin) (Yssel, et al 1984), supplemented with 10% foetal calf serum (FCS, Gibco/Amersham Life Sciences, Arlington Heights, IL) at 5% CO2. The basophilic human cell line KU812 (Kishi 1985) was cultured in RPMI medium supplemented with 10% FCS.

2.20 TRANSFECTIONS AND REPORTER ASSAYS.

The Transfection method used below was exactly as described in Nelms (1990).

GL-ε Promoter analysis (Figs. 6.12, 6.13): Log phase (≈$5x10^5$ cells/mL, $10x10^6$ total) BJAB, or BL-2 Lymphoblastoid B cells were washed once in phosphate buffered saline (PBS, Gibco/Amersham Life Sciences, Arlington Heights, IL) and resuspended in 0.75 mL TBS (for TBS, first two solutions were made: For solution A: 80 g/L NaCl, 3.8 g/L KCl, 2 g/L Na_2HPO_4, 30 g/L tris base, adjusted to pH 7.5 and filter sterilised. For solution B 15 g/L $CaCl_2$ and 10 g/L $MgCl_2$, were filter sterilised. 10 mL of solution A was added to 89 mL of dH_2O and 1 mL of solution B was added drop-wise with mixing.) containing 10 μg reporter plasmid, 6 μg RSV-β–galactosidase control plasmid (Maggie Walmsley, London, UK), 4 μg RSV-Luciferase control plasmid (Gill May, London, UK), and 250 μL of 2 mg/mL diethylaminoethyl cellulose (DEAE) dextran was added. Plasmids were prepared by two rounds of CsCl gradient purification. The transfection was incubated for 30 minutes at 37°C. After transfection, the cells were diluted with 13 mL of media, spun at 1,200 RPM for 5 minutes, split into 2 aliquots and cultured for 48 hours in complete medium, in the presence or absence of IL-4 (100 U/mL) and anti-CD40 mAb 626.1 (5 μg/mL). Each transfection was performed in duplicate. Cell extracts were prepared, and luciferase activity determined according to the Luciferase Assay System (Promega) protocol. The same cell extracts were used to determine β–galactosidase (β-gal) activity using the chemiluminescent Galacto-Light kit (Tropix, Bedford, MA). All readings were taken using a Lumat Berthold 9501 luminometer (Berthold, Bad Wildbad, Germany. The results obtained for each transfection were normalised to β-gal activity.

2.21 PBMC ISOLATION AND CELL CULTURE.

PBMC were isolated from heparinised blood on ficoll gradients. Blood was drawn into a 50 mL syringe, and immediately mixed with heparin. Blood was kept at room temperature until used. 12 mL of blood was diluted with 12 mL RPMI medium without FCS, carefully layered onto 12 mL of ficoll-hypaque (Pharmacia, Piscataway, NJ) and spun at 1600 RPM for 30 minutes, 22°C, without a brake. Aspirate top layer leaving 5

mL containing PBMC. The PBMC layer was carefully taken from the gradient with a pastette, avoiding ficoll layer and immediately mixed with 4 volumes of RPMI without FCS. The PBMC were next spun at 1500 RPM room temperature, 10 minutes and washed 2 more times in medium at 1200 RPM for 10 minutes. Stored on ice.

Cultures were initiated at 1.5 X 10^6/mL in complete Yssel's medium (Yssel *et al.* 1984; Claassen *et al.* 1990), supplemented with 10% FCS (Gibco/Amersham Life Sciences, Arlington Heights, IL). 1 mL of cells was cultured in a polystyrene round bottom 13 mL (Falcon #2059, Becton Dickinson, Franklin Lakes, NJ) or 0.4 mL in a 4 mL FACScan tube (Becton Dickinson, San Jose, CA.) at 5% CO_2. The cultures were not disturbed until 12-14 days later. Recombinant human IL-4 was a gift of DNAX (Palo Alto, CA, specific activity $1x10^7$ U/mg). Fluticasone, (a kind gift of Glaxo Welcome, Stevenage, UK), was dissolved at a concentration of 0.2 M in N, N-dimethyl-acetamide (Sigma, St. Louis, MO), and diluted appropriately in 0.9 M NaCl (physiological saline), 1% polyoxyethylenesorbitan monooleate (tween 80, Sigma, St. Louis, MO). IL-4 and fluticasone were added at the onset of culture.

2.22 EMSA PROBES AND COMPETITORS

The GL-ε 80-184 EMSA probe (Fig. 6.4) was generated from a 106 bp Xma I-Bcl I fragment cleaved from the gel isolated 36/266 PCR product (Table 6.1), and end labelled with Klenow polymerase and α^{32}P-dCTP (see 2.1 END LABELLING) and isolated on a 6% non-denaturing polyacrylamide gel (see 2.11 ACRYLAMIDE GEL ISOLATION). The GL-ε 42 base 36/78 probe was cleaved with XmaI from the 36/266 PCR product, end labelled with Klenow polymerase and α^{32}P-dCTP and isolated on a non-denaturing polyacrylamide gel. The 104/191 and 104/139 probes (Table 6.1) were generated by first isolating (on a 6% and 10% non-denaturing polyacrylamide gel, respectively), a single stranded template generated by asymmetric PCR with the upper strand at 1 μM and the lower strand at 0.05 μM. The second strand was synthesised with sequencing Taq polymerase (Promega Biotec, Madison, WI) in the presence of unlabelled dATP, dGTP, dTTP and (2 μL/20 μL total volume) α^{32}P-dATP (10 ci/mL, 3000 ci/mMol), and 1 μM and the lower strand, for one cycle at 95°C for 2 minutes, at 60°C for 5

minutes, at 72°C for 3 minutes, and chased with 0.4 µL unlabelled 10 mM dATP, and 0.5 µL Taq 5 units/µL. The labelled probes were then isolated on a non-denaturing acrylamide gel (6% and 10%, respectively).

Competition oligonucleotides were annealed in 100 mM NaCl, 10 mM Tris pH 8, 0.1 mM EDTA by heating to 95°C 10 minutes and cooling slowly to the annealing temperature and incubating for 1 hour. Mutant BSAP competition oligonucleotides were generated by PCR by using the BSAP1XMUT and BSAP2XMUT oligonucleotides. All probes and competitors were purified on polyacrylamide gels.

2.23 NUCLEAR EXTRACTS AND ELECTROPHORETIC MOBILITY-SHIFT ASSAY.

Nuclear extracts were prepared as follows: 2-3X10^6 cells were washed 2 times in phosphate buffered saline (PBS), resuspended in 400 mL of buffer A (10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, with the protease inhibitors: 0.2 mM phenylmethylsulphonyl flouride (PMSF), 1 mg/mL aprotinin, 3 mg/mL antipain, 3 mg/mL pepstatin, 3 mg/mL leupeptin and the phosphatase inhibitors: 1 mM benzamidine, 1 mM ortho sodium vanadate, 1 mM sodium fluoride and 5 mM b-glycerol Phosphate) and incubated on ice for 10 minutes. NP-40 (0 003-0.01%) was added if the nuclei were not released within 10 minutes. The nuclei were sedimented in a micro-centrifuged for 12 seconds at 4°C and then resuspended in 2 volumes of buffer C (20 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], 25% glycerol, 1.5 mM $MgCl_2$ 0.2 mM EDTA, 0.5 mM dithiothreitol, with protease and phosphatase inhibitors.). The total volume of the nuclei was measured and the nuclei were transferred to a new tube. Exactly 1 volume of buffer C containing 840 mM NaCl was then added, and the nuclei were extracted for 20 minutes on ice, with occasional mixing. The nuclei were sedimented at in a micro-centrifuge for 5 minutes at 4°C and the extracts were frozen on dry ice and stored at -70°C. Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL).

For EMSA, 5 mg of nuclear extract was added to 20 mL of binding buffer: 10 mM tris pH 8, 0.5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, with 5 mg poly(dI-dC)-

poly(dI-dC) (Pharmacia, Uppsala Sweden) and 5 fMoles of ^{32}P-labelled probe in the presence or absence of competitors. NaCl and glycerol were adjusted to 0.1 M and 10%, respectively. After incubation for 30 minutes on ice, the binding reactions were run on a 4% polyacrylamide gel in 50 mM tris, 50 mM boric acid, 1 mM EDTA pH 8, 4% glycerol at 30 mA for 2-5 hours at 4°C.

Supershifts were performed by preincubating the nuclear extracts in binding buffer with the appropriate antisera for 30 minutes on ice before adding the probe and competitors.

2.24 IgE ENZYME-LINKED IMMUNOABSORBENT ASSAY.

Materials: Coating antibodies consisted of fresh solution of 3 µg/mL each of Mouse IgG anti-human IgE mAb 7.12 and 4.14 (or 7.12 alone) (Macy *et al.* 1988) in 0.2 M carbonate buffer (3.73 g Na_2CO_3 with 3.76 g $NaHCO_3$ to 400 mL, pH 9.8 with HCl, 10 µg/mL sodium azide). Nunc 96 well immunosorb plates (Nalge Nunc Intl, Rochester, NY).

Method: Each well was coated with 50 µL the monoclonal antibodies 7.12 and 4.15, and incubated at 37°C for 1 hour (or room temp several hours or 4°C overnight). The plate was washed 2 times with Phosphate buffered saline (PBS). The wells were filled (≈200 µL) with 2% non-fat dried skim milk (Marvel, UK), and incubated at 37°C for 30 minutes to 1 hour (or room temperature for 2-3 hours). The plate was washed 3 times with PBS/0.05% Tween 20 (# P-1379, Sigma St. Louis, MO) and beat on towels. 50 µL supernatant (diluted in medium) to be analysed, or an IgE standard curve (11 2 fold dilutions from 100 ng/mL, throw away tip at each well) diluted in medium was added to the well. The plate was washed 4 times with PBS/Tween. The top layer antibody (1:1000 of #P295, Dako, High Wycombe) was diluted in 1% milk/PBS/Tween, 50 µL of the antibody was added per well and the plate was incubated at room temperature for several hours. The plate was washed 5 times with PBS/Tween, beat on a towel, washed 1X, and beat again. To each well was added 50 µL of developing solution (20 mM citric acid, 50 mM $NaHPO_4$ pH 5, 0.02% H_2O_2, 4 mM o-phenylenediamine (OPD; P-9029, Sigma, St. Louis, MO), the OPD was dissolved in few drops methanol, the buffer and

H_2O_2 were added, and it was used immediately). the plate was incubated at room temperature for at least 30 minutes or until the yellow colour developed. 50 µL 0.1 M H_2SO_4 was added to each well to stop the reaction, and the absorbance was read at an optical density of 492.

2.25 IMMUNOFLOURESCENT ANALYSIS OF CELL SURFACE ANTIGENS.

Membrane staining was performed using a panel of B cell-specific mAbs and differentiation specific mAbs to phenotypically characterised B cell lines and primary B cells taken from tonsil. These included MHC-class II (RFDR2γ), CD19 (RFB9γ), CD20-like (RFB7µ), CD38 (RF10γ or µ), CD21 (RFB6γ), kind gift of George Janossy (Royal Free Hospital, London, UK); CD23 (BU38γ), IgM (BU1γ); from The Binding Site (Birmingham, UK); and CD40, kind gift of E. Clark (Seattle, WA). Isotype specific second layer antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were added. One or two colour immunofluorescence was detected using a FACscan (Becton Dickinson, San Jose, CA.) and analysed using PC Lysis software.

2.26 TRANSCRIPTION FACTOR BINDING SITE ANALYSIS.

To find potential transcription factor binding sites, the Signal Scan suite of databases version 4.07 was used on the MRC-HGMP resource centre computer (Cambridge, UK). Signal Scan is a compilation of three databases (Prestridge 1991); TFD (Ghosh 1992), Transfact (Wingender 1988), and the Information Matrix Database Search (Chen *et al.* 1995). Only factors considered potentially relevant to the expression of the FcεRIα gene, or factors with matches of 6 or more invariant nucleotides, were chosen for inspection.

CHAPTER 3: Cloning of the Human IgE High Affinity Receptor (FcεRI) Gene.

3.1 INTRODUCTION

The cDNA's for the high affinity receptor alpha chain have been cloned for the rat (Shimizu *et al.* 1988; Tepler *et al.* 1989), mouse (Ra *et al.* 1989), and human (Kochan *et al.* 1988; Shimizu *et al.* 1988). The genes for the alpha chain have also been cloned for the rat (Tepler *et al.* 1989) and the mouse (Ye *et al.* 1992). Although the rat and mouse FcεRIα polypeptide chains are only 70% identical, the nucleotide sequences are 84% identical in the coding region, and 92% in the 5' untranslated region. This suggests that although the protein has diverged between the rat and mouse, the DNA sequence, especially in the region of the promoter, has had pressure to remain evolutionarily conserved. Likewise, the regulation of the gene has also probably remained highly conserved between the rat and mouse. In contrast, the human FcεRIα polypeptide chain is only 50% identical to the rodent, and 70% on the DNA level. Such divergence between the human and rodent on the nucleic acid level might suggest a divergence in regulation of the gene from what is seen in the rodent. Likewise, to understand the regulation of FcεRIα in the human, the gene was first cloned, and studied directly.

Little is known about the regulation of the FcεRIα gene. IFN-gamma and alpha/beta have been shown to increase FcεRIα mRNA levels in rat mast cell lines (Enciso *et al.* 1996). FcεRI levels on blood basophils has been shown to increase and decrease in a direct relationship with IgE levels (Lantz *et al.* 1997; MacGlashan *et al.* 1997; Yamaguchi *et al.* 1997). FcεRI increases have been shown to be dependent on protein synthesis, but whether this regulation is on a transcriptional, translational, or post-translational level is not known.

Genes are regulated on the transcriptional level at the promoter by two types of factors. First, general factors bind to RNA polymerase to assemble the pre-initiation complex, which forms around the start site of transcription, and is required for the initiation of transcription (for transcription review, see Orphanides *et al.* 1996; Roeder 1996; Lewin 1997). Upstream factors, both inducible and constitutive, compose the second group and modulate the efficiency of initiation.

The upstream region (Fig. 3.5) of the FcεRIα gene contains sequence identity to the binding sites for the general transcription factors TFIID and TFII-I. Most genes, except rRNA, tRNA, 5SRNA and snRNA, are transcribed by RNA polymerase II. The general factors that bind to polymerase II are termed TFIIX. Of this group TFIID binds to the TATA box, which is positioned normally 30 bases upstream of the transcription start site (Nussinov 1992), and promotes the assembly of the pre-initiation complex. Overlapping with the transcription initiation site in an initiator sequence. TFII-I binds to the initiator, and to TFIID, helping to position the start of transcription (Roy *et al.* 1991; Roy *et al.* 1993a). In promoters that lack a TATA box, TFIID binds at other less well defined sequences in a TFII-I dependent manner.

TFII-I is immunologically related to the ubiquitous basic-helix-loop-helix (bHLH) protein family member upstream stimulatory factor (USF, Roy *et al.* 1991). bHLH proteins are upstream regulatory factors that bind the consensus E-box CANNTG. Two potential E-boxes are present in the upstream region. TFII-I has been shown to interact co-operatively with USF, and another bHLH protein c-Myc (Roy *et al.* 1993b), in binding to both the E-box and the initiator element. TFII-I and USF interact co-operatively at both the initiator element and the E-box. TFII-I also interacts with c-Myc, a proto-oncogene expressed in most dividing cells (Zimmerman *et al.* 1986), interacting co-operatively at both the initiator element and the E-box (Roy *et al.* 1993b). However, in this case c-Myc inhibits transcription by preventing the interaction between TFIID, TFII-I, and the promoter. Both cases present a mechanism of communication between upstream regulatory factors and the general transcription machinery.

Several bHLH proteins potentially bind to the upstream sequences, including the ubiquitous c-Myc (Ayer and Eisenman 1993) and E2A (Visvader *et al.* 1991; Vierra *et al.* 1994), the skeletal muscle specific MyoD (Goldhamer *et al.* 1992; Miner *et al.* 1992) and myogenin (Wright *et al.* 1989), and AP-4 which is expressed in the myeloid lineage, among other cell types (Comb *et al.* 1988; Mermod *et al.* 1988; Gabuzda *et al.* 1989). Several other bHLH proteins are known to be expressed in the myeloid lineage including USF (Sirito *et al.* 1994), Mxi1 and Max (Zervos *et al.* 1993), Mad (Ayer and Eisenman 1993), tal-1(Voronova and Lee 1994; Condorelli *et al.* 1997), MITF (Tsujimura *et al.* 1996), ROX (Meroni *et al.* 1997) and may bind to one or both of the E-boxes as well.

bHLH proteins contain a HLH dimerisation domain which allows homodimers to form, and also heterodimers between members of the bHLH family. Some family members, such as c-Myc and USF belong to a second class, the bHLH-zip proteins, and can form higher order multimers by addition of the leucine zipper domain (Beckmann and Kadesch 1991). Interaction between bHLH family members can allow communication between regulatory elements within a promoter, and between a promoter and enhancer (Zimmerman *et al.* 1986; Roy *et al.* 1991; Artandi *et al.* 1994).

The upstream region of the mouse gene also contains a potential CCAAT box. When present, CCAAT boxes occur from 50 to 200 bases upstream of the transcription start site (Nussinov 1992). Nearby the homologous position in the human and rat to the mouse CCAAT box are sites for the CCAAT binding factors C/EBP (Johnson *et al.* 1987; Ron *et al.* 1990), and CTF/NF-1 (Morgan *et al.* 1987; Paonessa *et al.* 1988; Abraham *et al.* 1990; Graves *et al.* 1991). C/EBP is expressed ubiquitously (Landschulz *et al.* 1988; Wedel and Ziegler-Heitbrock 1995), and CTF/NF-1 is expressed in myeloid, adipocyte and hepatocyte lineages (Bienz 1986; Jones *et al.* 1987). The activation domain of CTF/NF1 and C/EBP interacts with TFIIB, helping recruit it to the preinitiation complex (Kim and Roeder 1994; Nerlov and Ziff 1995).

Also present is the potential binding site for LF-A1 (Ammendola *et al.* 1990), a liver specific regulatory factor for the NF-1 gene, and HNF-3 (Grange *et al.* 1991), which has binding sites that overlap with C/EBP in the rat aminotransferase gene. HNF-3 belongs to the HNF-3/fork head family of transcription factors which share homology in the winged-helix DNA binding domain (Lai *et al.* 1993), is expressed in the bronchial epithelium, lung, liver, stomach, small intestine, embryonic notochord, floor plate, and gut (Ang and Rossant 1994; Kaestner *et al.* 1994; Weinstein *et al.* 1994), and is involved in the inflammatory response in the lung (Chen and Liao 1993; Magdaleno *et al.* 1997).

Three potential GATA binding sites are present in the upstream sequences. GATA family members GATA-1, 2, and 3, are expressed in haematopoetic cells, and bind to the consensus sequence $^{A}/_{T}GATA^{A}/_{G}$ (Ko and Engel 1993; Merika and Orkin 1993). GATA-1 binds to a site in the chicken beta-globin promoter that includes the canonical TATA box, and a site in the beta-globin enhancer. The enhancer GATA-1 site can functionally replace the TATA box in the promoter. GATA-1 interaction with the TATA box is required for enhancer dependent

transcriptional regulation (Fong and Emerson 1992), suggesting that GATA-1 both aids in communication between an enhancer and promoter, and enables TFIID to be regulated by distal elements.

At position -71, a polymorphism containing either a T or C, was found in the FcεRIα gene at a potential GATA-1 binding site (Barnhart *et al.* 1989). This polymorphism has at least a slight linkage to atopy (D. Fear, pers. comm.). When a T is present at this position, two EMSA bands are seen, and only one is present in the C allele.

The sequences upstream of the coding region were analysed in luciferase reporter assays and were found to contain promoter activity in human basophils.

3.2 RESULTS

3.2.1 Cloning of the FcεRIα gene

Oligonucleotide sequences chosen to screen a genomic library were checked for potential cross hybridisation by searching against the Genbank/EMBL database of sequences (Wisconsin Package, Genetics Computer Group, Madison, WI).

For the first screening, an oligonucleotide specific for exon 4 of the human cDNA (Kochan *et al.* 1988; Shimizu *et al.* 1988), hFcεRI31 (Table 3.1; Fig 3.1) was used to probe 10^6 plaques of a λ-embl3 male human leukocyte genomic library (#HL1006d, Clonetech, Palo Alto, CA), infected into the *E. coli* bacterial strain LE392. The library was composed of Mbo I partial digest of genomic DNA constructed to allow excision of the entire insert by a Sal I digest. Five potential clones were identified. Four of the five potential clones were confirmed by a secondary screening with an oligonucleotide specific for exon 5, hFcεRI32. The four clones, 3C, 17, 22 and 28, contained inserts of approximately 15.9, 15.8, 15.7, and 18.1 Kb, respectively (Fig. 3.2). Each clone was additionally confirmed by DNA sequencing (for sequencing strategy see Fig. 3.1).

Table 3.1. Oligonucleotides used in cloning and analysis of the FcεRIα gene.

Name	Sequence	Loc[a]	Orient	Exon
5FC	CTGCTCTCCCTTGCATCC	-348/-331	+	
5'αR	CTGTGCTGGAGAGATCTAAGGCTTC	-50/-26	-	1
EX2F	CTCCAGATGGCGTGTTAG	116/133	+	2
EX2R	GAGCTAACACGCCATCTG	119/134	-	2
RIF	CCTCAGAAACCTAAGGTC	139/157	+	3
RIR	CTGAAGACTTCCAGGTAC	372/389	-	3
hFcεRI17	CCTTATAATAGATCACC	486/502	-	4
hFcεRI31	GAGAGCTTCACCATCCTTATAATAGATCACC	486/516	-	4
EX4F	CAGTGGAACCTACTACTG	570/587	+	4
hFcεRI32	CTGTGTCCACAGCAAACAGAATCACCACCAAC	690/721	+	5
hFcεRI16	GCAAATACCTCTTGAG	843/858	-	5

[a] Base number 1 is at the A in the translation start codon.

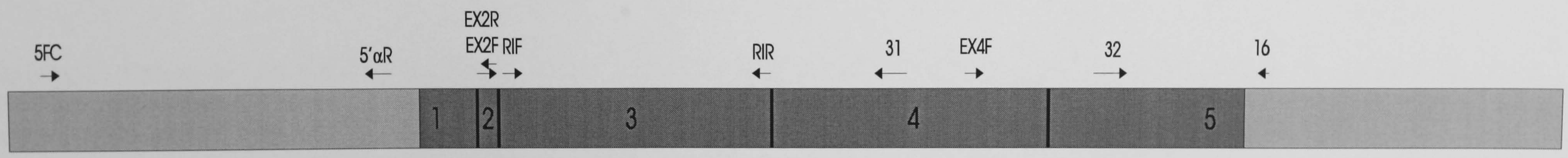


Figure 3.1. Location of oligonucleotides used for cloning and sequencing. Map of the FcεRIα cDNA, showing coding sequence (dark grey), 5' and 3' untranslated (light grey). Oligonucleotides (arrows) are shown above.

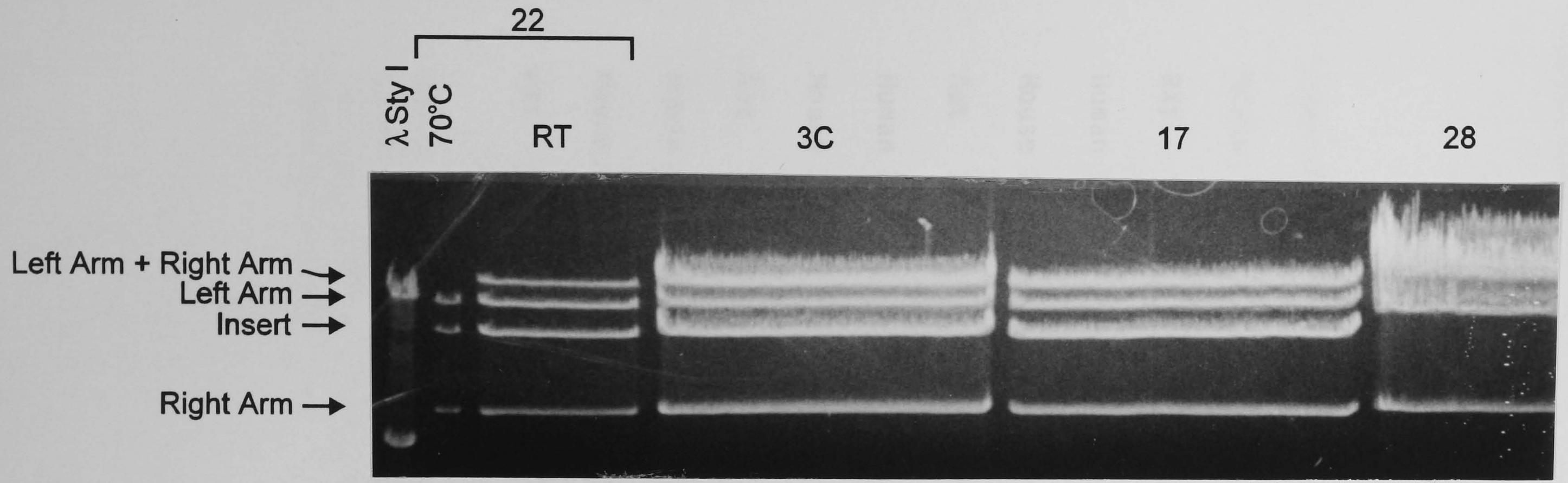


Figure 3.2. Sal I digest of FcεRI-α lambda clones. The right and left arms of the lambda EMBL3 cloning vector are separated from the insert containing the FcεRI-α gene by a Sal I digest. At room temperature (RT) the right and left arms anneal via the lambda COS site to form a dimer (right arm+ left arm), which is dissociated at 70°C. The 19.3 and 7.7 kb DNA fragments of Sty I cut λ-CI857 are used as a molecular weight marker (λ Sty I).

Intron sequences at the intron/exon border were identical to the rat, or mouse at 4 of the 8 borders (Fig. 3.3). At 90%, similarity between the human and rodent intron/exon borders was higher than the 70% homology in the coding sequence, demonstrating the importance of sequence conservation to the splicing machinery. All intronic sequence at the intron/exon border obeyed the GT.....intron.....AG rule (for splicing review, see Padgett *et al.* 1986).

```
Human 110 TCTTCGgtaagtagag/--intron 1--/acttttgcagCTCCAG 121
          || |  ||||||| ||                |||||||||||| |
Mouse  83 TCATGTgtaagtacag/--intron 1--/gattttgcagCTCCTG 94
          |||| ||||||||||||               ||||||||||||||  |
Rat    97 TCATATgtaagtacag/--intron 1--/gattttgcagCTCTGG 108

Human 131 TAGCAGgtgagtcctc/--intron 2--/cttcttgaagTCCCTC 142
          |  |||||||||                  |||||| ||| | ||
Mouse 103 TGACAGgtgagtgagt/--intron 2--/cttctttaagCCACTG 114
          | ||||||  |||                  ||||||||||||||||
Rat   118 TAACAGgtaagtcctg/--intron 2--/cttctttaagCCACTC 129

Human 386 TCAGTGgtaagttcca/--intron 3--/tgttcttcagACTGGC 397
               ||||  ||||||                || | ||||||  ||||
Mouse 362 CGCAAGgtacgttcca/--intron 3--/tgcttttcagATTGGC 373
           ||||||||||||||||                 ||||||||||  ||||
Rat   373 TGCAAGgtacgttcca/--intron 3--/cgcttttcagAGTGGC 384

Human 644 TAAAAGgtgagttggt/--intron 4--/tccactacagCT..C. 652
          ||||||||  |||||  |                ||||  |  ||||  |
Mouse 617 TAAAAGgtaagttgat/--intron 4--/tccattctagCTTACA 628
          ||||||||||||||||                 ||||||||||  |||||
Rat   631 TAAAAGgtaagttgat/--intron 4--/tccattctagATTACA 642
```

Figure 3.3. Sequence of Exon/intron borders. Uppercase, exon sequence, lowercase, intron sequence. Numbering refers to the human (Genbank: hsfceri), mouse (Genbank: musfcera), and rat (Genbank: ratmciaa) sequences.

The 5' untranslated region was sequenced to 349 nucleotides upstream from the transcription start site, and showed a moderate, 75% identity to the mouse and rat. This region is in agreement between all four clones, except for a C or T at nucleotide -71 (Fig. 3.4). Subsequent work by David Fear has shown this position corresponds to a polymorphism, with a slight linkage to atopy. The presence of the C or T allele was assayed in 15 atopic and 15 non-atopic individuals by sequencing. All 4 (2 homozygous and 2 heterozygous) of the 30 individuals containing a C allele were non-atopic.

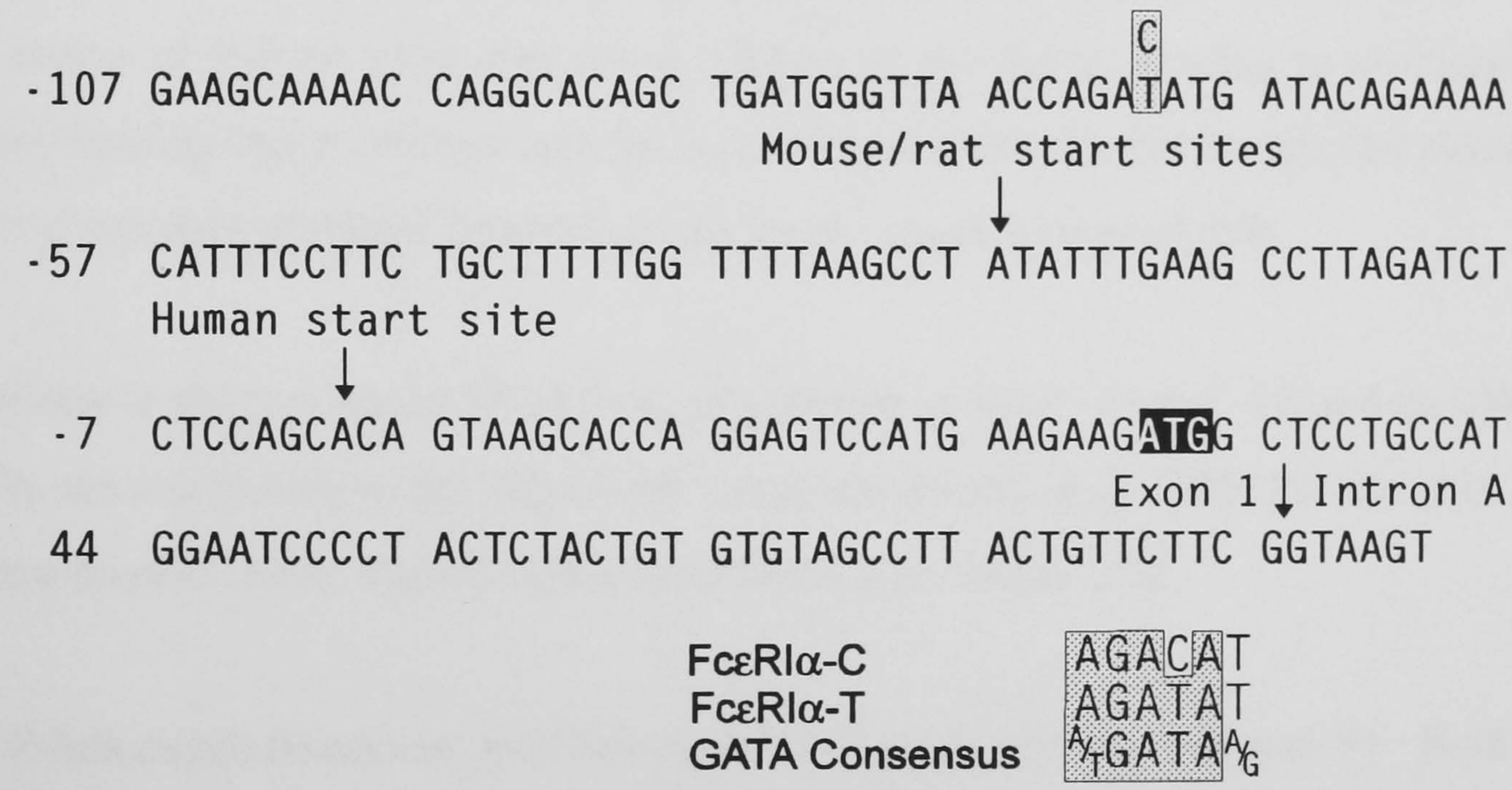


Figure 3.4. C/T polymorphism in the 5' untranslated region. Above, location of the polymorphism (grey box) at position -71, the relative position of the rat, mouse and human transcription start sites, and the translation start site (black box). Below, comparison of the two polymorphic sequences with the GATA consensus, and the region of homology (grey box) between the consensus and the FcεRIα sequences.

3.2.2 Potential transcription factor binding sites

A potential GATA-1 binding site (Barnhart *et al.* 1989) at the sequence AGATA (Fig. 3.4) is present in the T allele at position -74, and in conforms to the GATA consensus WGATAR (Plumb *et al.* 1989; Perkins *et al.* 1990; Yamamoto *et al.* 1990; Macleod and Plumb 1991) at 5 out of 6 positions. Although GATA-1 has not been shown to bind here by EMSA analysis (D. Fear, pers. comm.), two EMSA bands are seen with the T allele, and only one with the C

allele. In addition to the potential GATA-1 site at position -74 is a second site immediately adjacent at position -69 (Barnhart *et al.* 1989). The presence of two EMSA bands in the AGATA allele and one in the AGACA allele may correspond to occupancy of the two adjacent GATA sites in the T allele, and only one in the C allele.

Sequences 5' of the coding region were searched for homology to other known transcription factor binding sites to look for likely regulatory elements in the promoter (see in Materials and Methods, 2.26; Table 3.2; Fig. 3.5). Regions of interest correspond to potential binding sites of at least 6 bases in length, and representing factors likely to be present in myeloid cells. Other potential binding sites representing related factors overlapping or immediately adjacent to a region of interest were also noted. Some of the factors binding to overlapping or adjacent binding sites mentioned may not be expressed in the myeloid lineage, but indicate the potential presence of related factors that may be expressed in myeloid cells.

In addition to the two partial WGATAR sites present at bases -74 and -69, and an additional GATA site conforming to the WGATAR consensus (Plumb *et al.* 1989; Perkins *et al.* 1990; Yamamoto *et al.* 1990; Macleod and Plumb 1991) is present at -122.

Two E-box motifs (Blackwell and Weintraub 1990) occur at bases -144 and -91. Both of the E-box motifs are immediately surrounded or overlap with several other potential binding sites. E-box motifs bind proteins of the basic-helix-loop-helix (bHLH) family. The distal E-box potentially binds the bHLH protein c-Myc (Suen and Hung 1991), and the proximal box potentially binds AP-4 (Comb *et al.* 1988; Mermod *et al.* 1988; Chu *et al.* 1991).

Additional bHLH proteins potentially bind at sites overlapping and adjacent to the proximal E-box motif. Immediately adjacent to the proximal E-box is a potential binding site for the bHLH protein myogenin (Baldwin and Burden 1989). Overlapping with both the proximal E-box and the myogenin site, are potential binding sites for two additional bHLH proteins MyoD (Rosenthal *et al.* 1990) and E2A (Xiao *et al.* 1987). The proximal E-box also overlaps with the potential binding site for the factor IUF-1 (Shelton *et al.* 1992; Bretherton-Watt *et al.* 1996), that is thought to be associated with the bHLH family member USF in the islet amyloid polypeptide (IAPP) promoter.

Table 3.2. Potential transcription factor binding sites found in the FcεRIα upstream sequence.

Factor	Lineage Specificity	Location Human	Location Rat	Location Mouse	Binding Sequence	Reference
C/EBP Family	adipocyte, hepatocyte, monocyte, macrophage	-137/-142 (-)	-110/-115 (+) -111/-119 (-)	-112/-120 (-)	CCCAAC TKNNGYAAK	(Johnson *et al.* 1987) (Ron *et al.* 1990)
CTF/NF-1	Ubiquitous		-105/-109 (+) -102/-106 (-)	-111/-115 (+) -106/-110 (+) -105/-110 (+) -105/-109 (+)	CCAAT TTGGC TTGGCA TGGCA	(Morgan *et al.* 1987) (Graves *et al.* 1991) (Paonessa *et al.* 1988) (Abraham *et al.* 1990)
HNF-3	Lung, Bronchial epithelium, Liver, stomach, small intestine, presumptive vertebrate head, neural tube		-103/-107 (+)		GGCCA	(Grange *et al.* 1991)
LF-A1	Liver	-144/-148 (-) -135/-139 (+)	-113/-117 (-)	-114/-118 (-)	GGGCA	(Ammendola *et al.* 1990)
Myb Family	Ubiquitous	-139/-144 (+)	-109/-113 (+)		CAGTTG CAACT	(Sureau *et al.* 1992) (Howe *et al.* 1990)
c-Myc	Ubiquitous	-139/-144 (+)			CAGTTG	(Suen and Hung 1991)
Myogenin	Skeletal Muscle	-92/-98 (-)	-65/-71 (-)	-65/-71 (-)	TGCCTGG	(Baldwin and Burden 1989)
MyoD	Skeletal Muscle	-86/-95 (-)	-59/-68 (-)	-59/-68 (-)	$CAGC(N)_5C$	(Blackwell and Weintraub 1990; Rosenthal *et al.* 1990)
AP-4	Myeloid, Brain	-86/-91 (+/-)	-59/-64 (+/-)		CAGCTG	(Comb *et al.* 1988; Mermod *et al.* 1988; Gabuzda *et al.* 1989)
E-box	Ubiquitous	-86/-91 (+/-)	-59/-64 (+/-)		CANNTG	(Blackwell and Weintraub 1990)
E2A	Ubiquitous	-86/-92 (+)	-59/-65 (+)		RCAGNTG	(Blackwell and Weintraub 1990)
IUF-1	Pancreas	-83/-88 (-)	-56/-61 (-)	-56/-61 (-)	CATCAG	(Shelton *et al.* 1992)
GATA-1	haematopoetic	-70/-74 (+)	-43/-47 (+)		AGATA	(Barnhart *et al.* 1989; Plumb *et al.* 1989; Macleod and Plumb 1991)
GATA-1	haematopoetic	-65/-69 (+)			TGATA	(Barnhart *et al.* 1989; Yamamoto *et al.* 1990)
GATA-1,2,3	haematopoetic	-117/-122 (+)	-88/-93 (+)	-88/-93 (+)	WGATAR	(Perkins *et al.* 1990; Yamamoto *et al.* 1990; Ko and Engel 1993; Merika and Orkin 1993)
TFIID	Ubiquitous	-19/-24	4/9		TTCAAA	(Tamura *et al.* 1990)
TFII-I	?			15/22	AGCTCTCT	(Roy *et al.* 1991)

K = G or T, Y = C or T, R = G or A, W = A or T

```
Human -349 ----------------------------TATTCAGTAATTAGTTGCTGCT -328

Human -327 GTTTTATTCTGCTCTCCCTTGCATCCCACTTTTCTAAGTTGTAAACTAAA -278
Rat   -291 ....C.....A.AG...TC.TT.CT..GTGG..TC....CTC...TC... -242
Mouse -265 ----------------------------.G..T.....CTC...TC... -245

Human -277 TAGTTGTACACAGATTG-ACAGATTA---AGAAAGGCTTGTGATTGTGC- -233
Rat   -241 .TC...GG...G..AC.TT..CC..........TC.ACA...C.C...T -195
Mouse -244 .TC....G..TT..A..-T..CC...AGG..G..TC.ACA...C.C...T -196

Human  232 TAGACCTATGCCTCTCTCTCACCAGATTCCAGGTGTATATGTGGAGGTGG -183
Rat   -194 ...G..CT..GT....A.CT.........T.......-C.GC..A....AC -146
Mouse -195 ......CT..GTC.CTG.CT.T.......T.......-C.GC..A....AC -147

Human -182 GATAGGGAGTGGAGTAAGTGGGTAAATATTAAATTGCCCAGTTGGGCACC -133
Rat   -145 A..C----------.A..A.....G.C...A..........AC.T..... -104
Mouse -146 A..----------.A..A.....G.C...A..........A..T....A -103

Human -132 ATCCTGAATATTATCTCTAAAGAAAGAAGCAAAACCAGGCACAGCTGATG  -83
Rat   -103 .A..CT.T..........GG.......--C...GC..............  -56
Mouse -102 .C....-.T..........GG.......--C...GC........T.....  -56

Human  -82 GGTTAACCAGATATGATACAGAAAACATTTCCTTCTGCTTTTTGGTTTTA  -33
Rat    -55 A..........G.....G............C...CG......C.      -6
Mouse  -55 A.........CA.C.....G............C....G......C.      -6

Human  -32 AGCCTATATTTGAAGCCTTAGATCTCTCCAGCACAGTAAGCACCAGGAGT   18
Rat     -5 ......CT.....G...-...A.TG.TG....T......T.A..G     43
Mouse   -5 .......T..C......A.........TG.TG....T......T.A..G  43

Human   19 CCATGAAGAAGATGGCTCCTGCCATGGAATCCCCTACTCTACTGTGTGTA   68
Rat     44 .ACA.GG.C.-....A.A..-------.G.GGAT..G.C.GG.....CC..  86
Mouse   44 TGCA.GG.CG-....TCA..-------.G.AGGT..G.C.AG.....CC..  86
```

C/EBP/CTF/NF-1/HNF-3/LF-A1
c-Myb/E-box c-Myc CCAAT
GATA
GATA GATA
+1
+1
TFIID
TFII-I
Met
MyoD/myogenin/E2A
c-Myb/AP-4/E-box/IUF-1

Figure 3.5. Location of potential transcription factor binding sites in the region upstream of the FcεRIα gene. The 5' regions of the human, rat and mouse genes are shown. Nucleotides of the rat and mouse genes similar to the human gene are represented as a period and gaps created for sequence alignment as a dash. Regions of homology to known transcription factor binding sites are boxed in the 5' regions of the human, rat, and mouse genes. An inverted repeat surrounding bases -141/-142 in the distal grouping of binding sites in the human, is marked with arrows. The start of translation, Met, is indicated in blue. The transcription start sites in the mouse and rat (blue arrow), and human (black arrow) are indicated.

Both E-box motifs also potentially bind the non-bHLH protein c-Myb (Sureau *et al.* 1992) with the distal site a perfect match, and the proximal site slightly unfavoured for c-Myb at the G in position 3 (Deng *et al.* 1996).

In the mouse sequence a CCAAT box overlaps with the distal E-box. A number of factors that are known to bind the CCAAT box also have potential binding sites in or near the E-box/CCAAT box motif. The CCAAT/enhancer-binding protein (C/EBP) family (Johnson *et al.* 1987; Landschulz *et al.* 1988; Brasier *et al.* 1990; Ron *et al.* 1990; Ron *et al.* 1991; Brasier and Kumar 1994) and NF-I (Paonessa *et al.* 1988; Abraham *et al.* 1990; Graves *et al.* 1991) potentially bind sites overlapping or adjacent to the region that contains the CCAAT motif in the mouse. Both the C/EBP family and NF-1 are known to bind to CCAAT boxes, as well as other sequences, though interestingly the corresponding human and rat regions do not contain a CCAAT motif. LF-A1 (Hardon *et al.* 1988; Ammendola *et al.* 1990) also binds in this region, and is known to regulate NF-1. HNF-3 (Grange *et al.* 1991), binds here as well, and is known to have a binding site that overlaps with various C/EBP sites in the rat tyrosine aminotransferase gene.

Adding to the possibility that a regulatory element resides at the E-box/CCAAT motif, a perfect palindrome with 6 base arms is present overlapping the human E-box/CCAAT motif, with near perfect palindromes in the corresponding rat and mouse sequences. The palindrome is centred in the E-box, extending 4 bases beyond each side of the E-box. Many regulatory elements, including the E-box, are known to be composed of two half-sites, often present as a palindrome, and represent the binding of two half sites by a regulatory factor.

No TATA box is found near to the mouse (Ye *et al.* 1992) rat (Tepler *et al.* 1989) and human (D. Fear, pers. comm.) transcription start sites, suggesting a TATA-less promoter, though no multiple GC box is found, an element commonly present in TATA-less promoters.

A potential TFIID site (Tamura *et al.* 1990), present in both TATA containing and TATA-less promoters (Roy *et al.* 1991; Roy *et al.* 1993a), is found near the start site in the human and rat sequences at position -24. A potential TFII-I binding site (Roy *et al.* 1991), also used in TATA-less promoters (Roy *et al.* 1993b; Manzano-Winkler *et al.* 1996; Montano *et al.* 1996), appears nearby the start sites at position -13.

3.2.3 Promoter analysis

Promoter fragments from the lambda clone 3C were identified and inserted into the reporter plasmid pGL2. Bgl II and Nco I were chosen as restriction site termini for the promoter fragments because both sites are near the 3' end of the first exon of the FcεRIα gene (Fig 3.7), insuring that all potential promoter sequences would be included in the expression constructs. Bgl II and Nco I fragments from containing the putative promoter region were identified from clone 3C by a polymerase chain reaction (PCR) based method. Clone 3C was digested with Bgl II or Nco I and the fragments were isolated from an agarose gel (Fig. 3.6). Restriction fragments containing the putative promoter region were identified by PCR using the oligonucleotides 5αR and 5FC (Fig. 3.6, Table 1). A 1.2 Kb Bgl II fragment and a 4.0 kb Nco I fragment were identified to contain the putative promoter fragments. The Nco I fragment containing the putative FcεRIα promoter, from -4000 to +45 was inserted into a reporter construct (Fig. 3.7) in both a forward and reverse orientation. Transient transfections were performed in the basophilic cell line KU812 (Kishi 1985). A 12 fold increase in expression from the putative promoter fragment was observed when compared to the same fragment in the reverse orientation (Table 3.3, and Fig. 3.8). Surprisingly, when a shorter 1.25 kb Bgl II fragment was used, expression was greater in the reverse orientation. After 3 transfections, and confirmation of the orientation by sequencing and restriction analysis, this result was reproducible. When both alleles of the two regions were re-cloned again, deleting all extra plasmid derived polylinker sequences generated during subcloning, the same result was observed (D. Fear, pers. comm.).

3.3 DISCUSSION

The human FcεRIα gene displayed a moderate degree of homology to the mouse and rat genes in the 5' untranslated region (75%) and a high homology (90%), in the intron/exon borders.

A polymorphism exists at nucleotide -71, either a C or a T occurring at this position. The C allele has been found to exist only in non-atopics, of 4 individuals in 30 found to contain at least one C allele. Analysis of a larger sample size will be required to confirm that the polymorphism is linked to allergy. But as the C allele was not present in atopics, therefore the potential role of this region of the promoter in the allergy is intriguing.

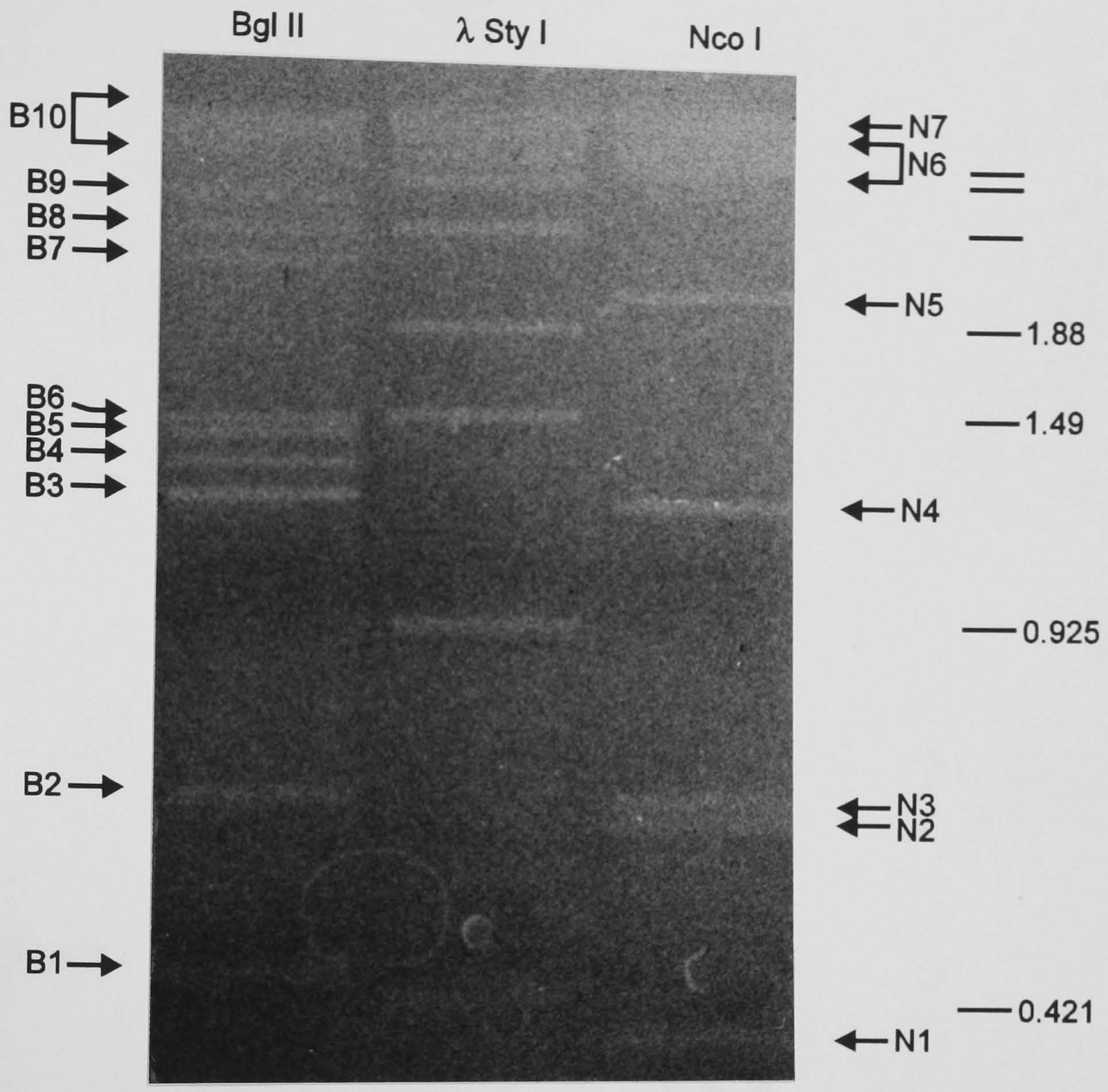


Figure 3.6. Isolation of the FcεRIα upstream sequences. (*A*), Preparative gel of the Bgl II and Nco I digests of FcεRIα clone 3C. 10 Bgl II and 7 Nco I bands (arrows) were isolated from the gel for identification by PCR and subcloning. The molecular weight marker (λ Sty I: λ-CI857 cut with Sty I) sizes are shown by the horizontal lines. (*B*) PCR mediated identification of the FcεRIα upstream sequences using the oligos 5'αR and 5FC (Table 3.1). The Bgl II fragment 3 and the Nco I fragment 6 were positive for the upstream sequences. (M), the molecular weight marker Bluescript cut with Hpa II.

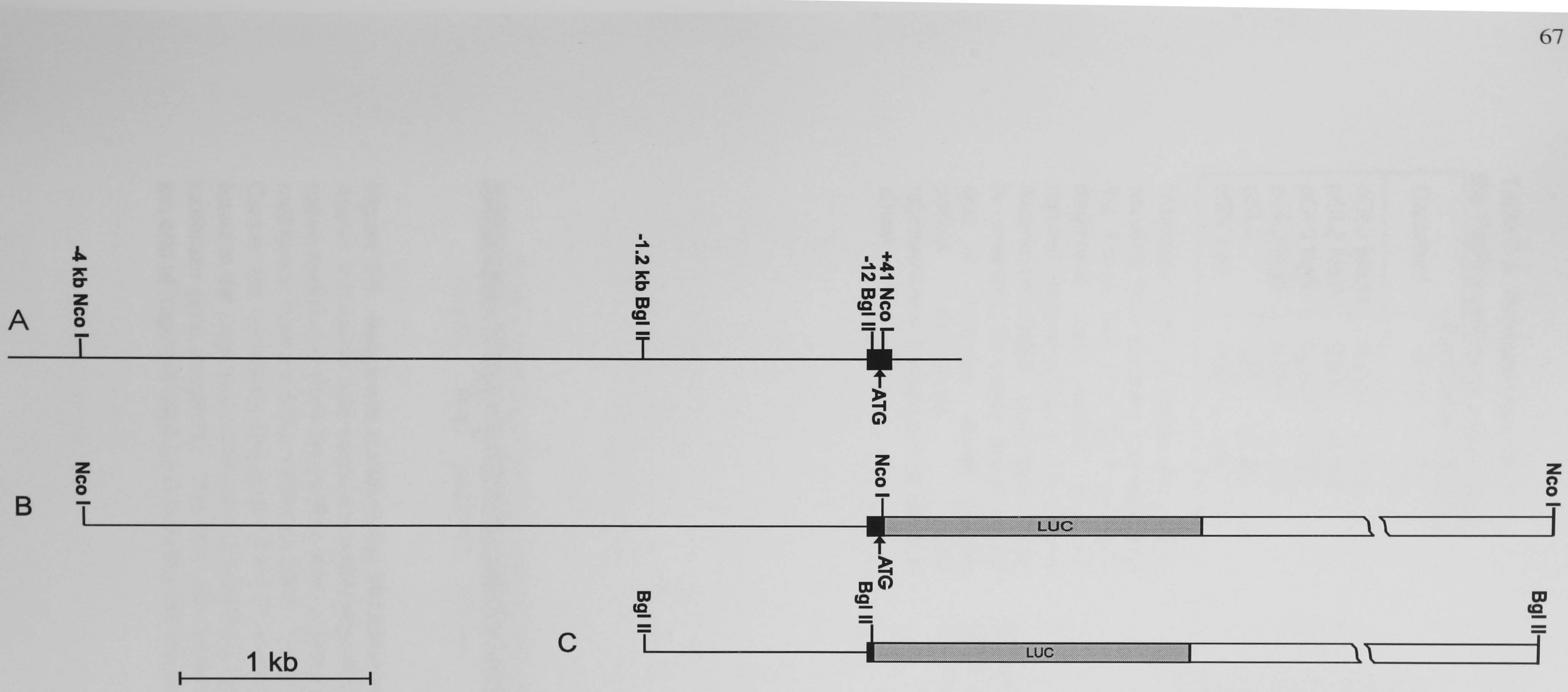


Figure 3.7. Luciferase constructs containing FcεRIα promoter fragments. **A**, Map of the region upstream of the FcεRIα gene, showing the first exon (black box), restriction sites and translation start codon (ATG). **B**, The construct containing the 4.0kb Nco I promoter fragment fused to the Luciferase reporter gene (grey box), and pGL2 vector sequences (open box). **C**, The reporter construct containing the 1.25 kb Bgl II fragment fused to the luciferase reporter vector.

Table 3.3. Promoter activity of the FcεRIα upstream sequences.

Construct	*Luciferase Activity*[a] *24 hr*	*48 hr*
pGL2 NcoI+	0.026	0.110
pGL2 NcoI-	0.006	0.007
pGL2 BglII+	0.024	0.031
pGL2 BglII-	0.097	0.134
pGL2	.005	0.05
RSV Luc	>90	>90

[a]Absolute units of Luciferase activity resulting from transient transfection of the 4.0 kb Nco I or Bgl II promoter fragments were inserted into the reporter plasmid pGL2, in a forward or reverse orientation. Luciferase activity is compared to vector alone (pGL2) and an enhancer driven positive control (RSV-Luc). One representative experiment of three is shown.

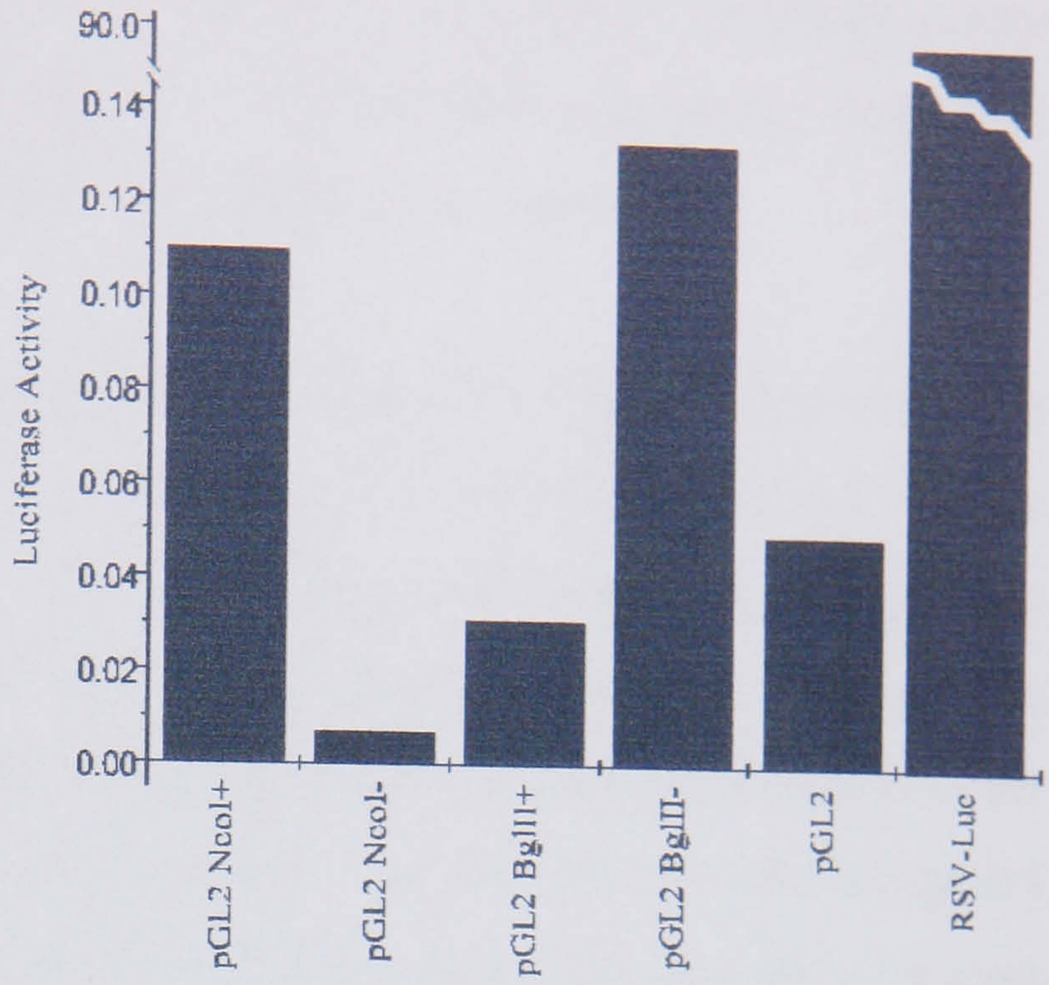


Figure 3.8. Promoter activity of the FcεRIα upstream sequences. Results of Table 3.3 expressed in graphical format.

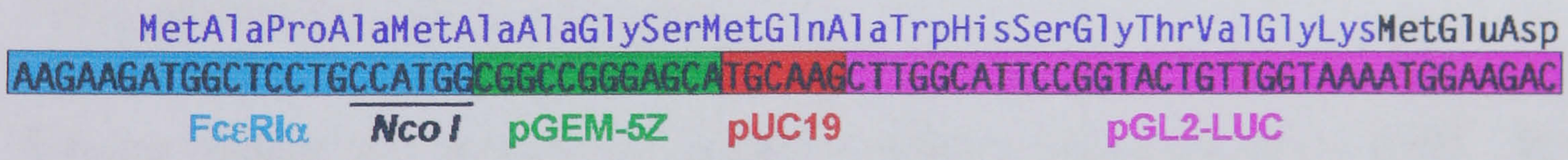


Figure 3.9. Sequence surrounding Met codons in the luciferase reporter construct. Above, the amino acid sequence potentially added (blue) to the luciferase protein if the native methionine from the FcεRIα gene is used in the reporter construct instead of the methionine from the pGL2-luciferase gene. The pGL2-luciferase protein is shown in black. Centre, the nucleotide sequence of the 3' end of the FcεRIα promoter fragment (cyan), fused to the polylinker sequences of pGEM-5Z (green), and pUC19 (red), and the pGL2-luciferase gene (magenta). The Nco I site (underlined) in the FcεRIα gene contains is at the end of fragment used for subcloning into the reporter construct.

Subsequent to this work, Pang *et al* (1993) published the a sequence of the FcεRIα gene which was in agreement with ours at all positions. The sequence reported by Pang *et al* was the T allele in the polymorphism found in this chapter at position -71.

The sequence TGATA at position -74 in the T allele is a potential GATA binding site. This sequence becomes TGACA in the C allele, and would likely reduce binding of GATA to the sequence. Although GATA has not been shown to bind to this sequence, EMSA analysis shows that two proteins of 50 and 100 kd bind here in the T allele, and only the 50 kd protein binds in the C allele. This pattern of binding could indicate that a protein binds to each of the two potential GATA sites surrounding the polymorphism. The fact that the 100 kd species could indicate binding of two identical 50 kd proteins also supports the idea that a protein binds to each GATA site. In addition to the two tandem GATA sites at -74 and -69 is another potential GATA site at position -122, that conforms to the well known WGATAR consensus sequence.

Two E-box motifs are present at bases -144 and -91. Multiple E-box elements are involved in the function of a number of regulatory elements; including the immunoglobulin enhancer (Ernst and Smale 1995), the immunoglobulin LCR (Meyer *et al.* 1995), and HIV-1 (Ou *et al.* 1994). bHLH proteins bind to E-boxes, can form heterodimers with other family members, and may provide physical bridges between control regions, (Artandi *et al.* 1994; Ferre *et al.* 1994). Either of the E-boxes may mediate interactions within the promoter, or with distant regulatory elements. Some of these interactions may be with the pre-initiation complex as is shown in associations between TFII-I and c-Myc (Roy *et al.* 1993b) or USF (Roy *et al.* 1991), affecting the efficiency of initiation.

E-boxes mediate both positive and negative regulation (Ernst and Smale 1995). In the immunoglobulin enhancer, the E-boxes μE4 and μE5 mediate repression, whereas μE2 and μE3 are involved in activation. At least one bHLH protein potentially binding here, MyoD, mediates chromatin reconfiguration in skeletal muscle genes (Gerber *et al.* 1997), and is probably involved in transcriptional activation in part through this mechanism. Although MyoD is expressed in the myogenic lineage, other related bHLH proteins binding to this site, such as c-Myc, AP-4 and E2A may perform the same function in myeloid cells. Transcriptional repression by at least one bHLH protein expressed in the myeloid lineage, MAD, is also mediated by chromatin reconfiguration through histone deacetylation (Laherty *et al.* 1997).

CHAPTER 4: Analysis Of Myeloma U266 Switch Recombination Junctions.

4.1 INTRODUCTION

During an immune reaction, antigen presenting cells display processed foreign antigen to B-lymphocytes in the lymph nodes (for review, see Kosco 1991; Neefjes and Momburg 1993). IgM bearing B-cells recognise antigen in the context of class II MHC and the resting B-cell becomes activated. Activated B-cells can switch the heavy chain component of an antibody to a downstream isotype, thus changing the effector function of the antibody without changing the antigen specificity (Reviewed in Vercelli and Geha 1992; Coffman *et al.* 1993). B-cell lines as well as B-cells isolated from peripheral blood, spleen and tonsil can be induced with combinations of lymphokines and mitogens to switch to IgG, IgE or IgA. In the human, primary B cells can be induced to switch to IgE and IgG4 in the presence of IL-4 and a second, cell contact mediated signal provided by activated T cells (Pene *et al.* 1988; Vercelli *et al.* 1989; Gascan *et al.* 1991b), or granulocytic cells (Gauchat *et al.* 1993). The cell contact mediated signal can be mimicked by cross-linking CD40 on the surface of the B cell (Gascan *et al.* 1991a), or addition of Epstein-Barr virus (Jabara *et al.* 1990), which may use a similar pathway of signal transduction as CD40 (Mosialos *et al.* 1995).

Leukaemic B-cell lines that express IgG, IgE or IgA show a deletion of the immunoglobulin heavy chain locus (for review, see Coffman *et al.* 1993). Deletion events occur between switch regions, located 1-2 kb upstream of each immunoglobulin heavy chain (IgH) constant region locus except Cδ. Switch regions contain highly repeated DNA sequence elements, and show some homology to each other. During the proposed mechanism for deletional recombination (Honjo and Kataoka 1978; Jack *et al.* 1988), the association of two switch regions allows the looping out and ligation of the intervening DNA to form an extrachromosomal circle.

Circular reciprocal recombination products have been isolated from B cells undergoing heavy chain switching (Iwasato *et al.* 1990; Matsuoka *et al.* 1990; von Schwedler *et al.* 1990; Yoshida *et al.* 1990). Evidence for successive switching from μ→γ4→ε and μ→α1→γ4→ε has been suggested from circular recombination products. Sγ4/Sε and Sα1/Sγ4/Sε junctions have been cloned from circular reciprocal recombination products, suggesting that some cells giving rise IgE have previously switched to IgG4 or IgA1 and IgG4 (Zhang *et al.* 1994).

In transgenic mice, heavy chain recombination can occur between a VDJ-μ construct and the endogenous γ-constant region on a different chromosome (Gerstein *et al.* 1990). This suggests that switch recombination can occur between homologous chromosomes, though no evidence yet exists showing this happens in normal switching events.

Recombination activity exists not only between, but also within switch regions. Deletions in the mu switch region have been found in subsequent switch junction analyses (Shapira *et al.* 1991). Deletions in switch regions have been shown to occur after the initial recombination event (Zhang *et al.* 1995), and have been postulated to be involved in a mechanism of isotype stabilisation, by reducing the size of the switch region remaining. Deletions within the μ switch region have also been found to occur prior to switching in Namalwa (Middleton *et al.* 1991), a cell line that has not undergone a heavy chain switching event. Internal deletions in Sμ also occur in mice deficient in switching (Cogne *et al.* 1994), due to deletion of the 3'IgH enhancer, further suggesting that recombination activity exists in the mu switch region in the absence of switching.

In chapter 4 the sequence of the switch recombination junction of the IgE secreting myeloma U266 (Nilsson *et al.* 1970; Nilsson 1971) is revealed. U266 appears to be the product of a sequential μ→α1→ε series of switching events. In the mu and epsilon switch regions two prominent pentamers, GAGCT, and GGGCT, were demonstrated to be highly repeated on the top strand of each switch region, and are probably involved in the mechanism of recombination.

4.2 RESULTS

4.2.1 Sequence of the U266 recombination switch junction.

Our objective was to map the location of a μ→ε recombination event by sequencing a switch recombination junction clone. We chose the IgE secreting myeloma U266 (Nilsson *et al.* 1970; Nilsson 1971) as a source of the Sμ/Sε switch recombination product. The clone λε1.2 (Fig 4.1, Flanagan and Rabbitts 1982), originating from U266, contains a rearranged epsilon locus (Flanagan and Rabbitts 1982). Comparison of the restriction enzyme map of

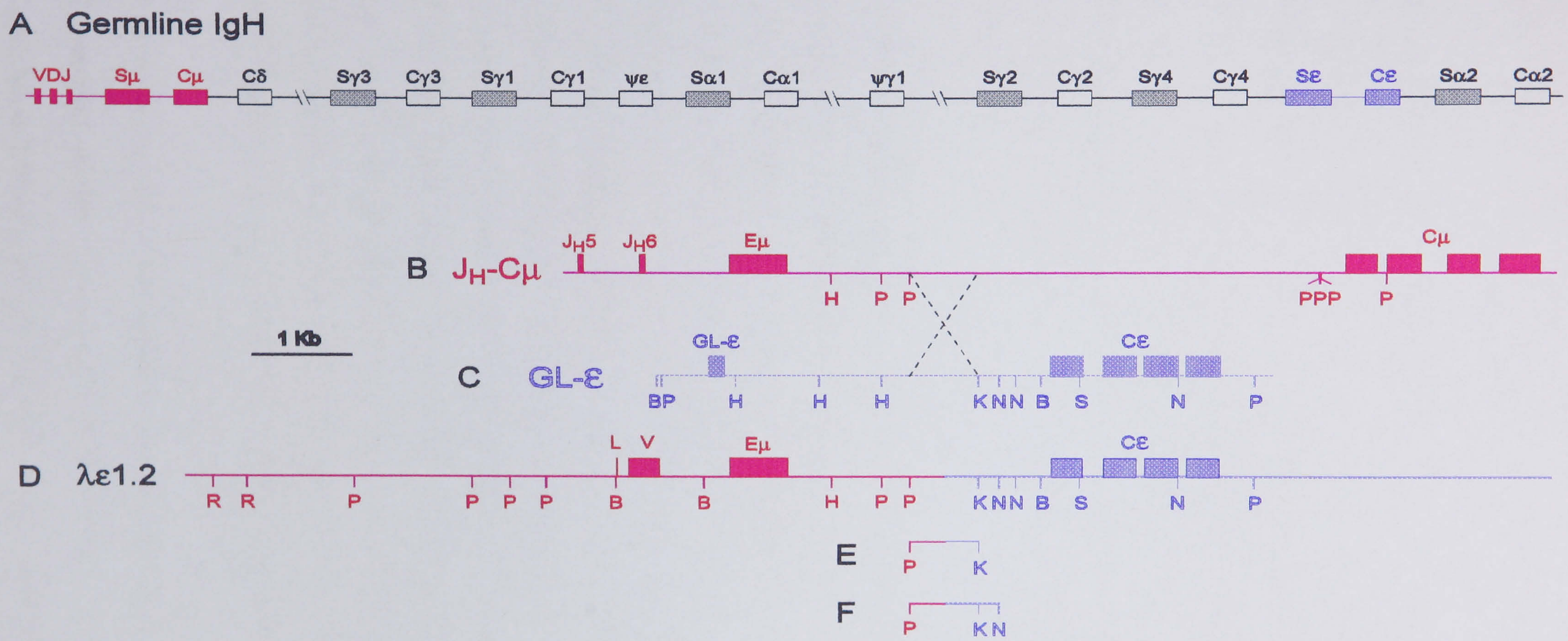


Figure 4.1. Map of clone λε1.2 (*A*) Map of the immunoglobulin locus. (B) Map of the germline J_H-Cμ region (red), (*C*) map of the germline epsilon locus (blue), and (*D*) the clone λε1.2. (*E*) (*F*) fragments used for sequencing the U266 switch recombination junction. The germline epsilon and mu maps are aligned to the regions of homology with the λε1.2 clone based on restriction fragment similarities. The map of the J_H-Cμ region (*B*) shows the variable region prior to V region assembly, whereas the V region of the λε1.2 clone has undergone recombination. B, BamHI; H, HindIII; K, KpnI; N, NcoI; P, PstI; S, SacI.

λε1.2 with that of the germline mu (Buluwela *et al.* 1988), and epsilon (Max *et al.* 1982; Ueda *et al.* 1982; Mills *et al.* 1990) loci suggested that the switch recombination event occurred within a 0.6 kb PstI-KpnI fragment (Fig. 4.1). Both the PstI-NcoI fragment, and a 0.8 kb PstI-KpnI fragment were subcloned from λε1.2 into bluescript and sequenced.

Sequencing from the 3' PstI site in Sμ revealed a 252 base deletion from nucleotides 3781-4033 of Sμ (see box I, Fig. 4.2). A second deletion of 1640 bases occurred in Sμ at nucleotides 4220-5860 (see box II, Fig. 4.2). Between the Sμ/Sε recombination junction is a 28 nucleotide fragment originating from position 1265-1292 of Sα1 (see box III, Fig. 4.2). The presence of a Sα1 fragment between Sμ and Sε in the region of the recombination join suggests that U266 switched to IgA1 prior to the expression of IgE (Fig. 4.3C). The switch recombination event to IgA1 to IgE occurred at base 3173 of the germline epsilon sequence (see box IV, Fig. 4.2).

Significant sequence homology exists between the switch regions that gave rise to the internal Sμ deletions (bases 3781-4033 and 4220-5860), and the Sμ/Sα1 switch recombination event are (Table 4.1; Appendix 1-3). Because of the homology between the switch region sequences, it was not possible to determine the exact location of the recombination events resulting in the Sμ deletions and the μ→α1 switching event. The homology between each switch region that underwent a deletion or switch recombination event suggests that semi-homologous pairing of switch regions contributes to the mechanism of switch recombination. Homology between Sε and both Sμ and Sα1 was low (Table 4.1; Appendix 4), suggesting that although homology between switch regions may play a role in the mechanism of switch recombination, a high degree of homology is not a requirement. Subsequently published Sμ/Sε junctions (Shapira *et al.* 1992; Zhang *et al.* 1994) demonstrated homology at the site of recombination between Sμ and Sε in other switching events (Fig. 4.4).

The deletion of Sμ bases 3781-4033 and 4220-5860 could have occurred at any time in the sequence of switching events. Because of the high degree of homology within Sμ and between Sμ and Sα, and the significantly lower homology between these regions and Sε, the 4220-5860 deletion event probably occurred some time before the μ→ε switch.

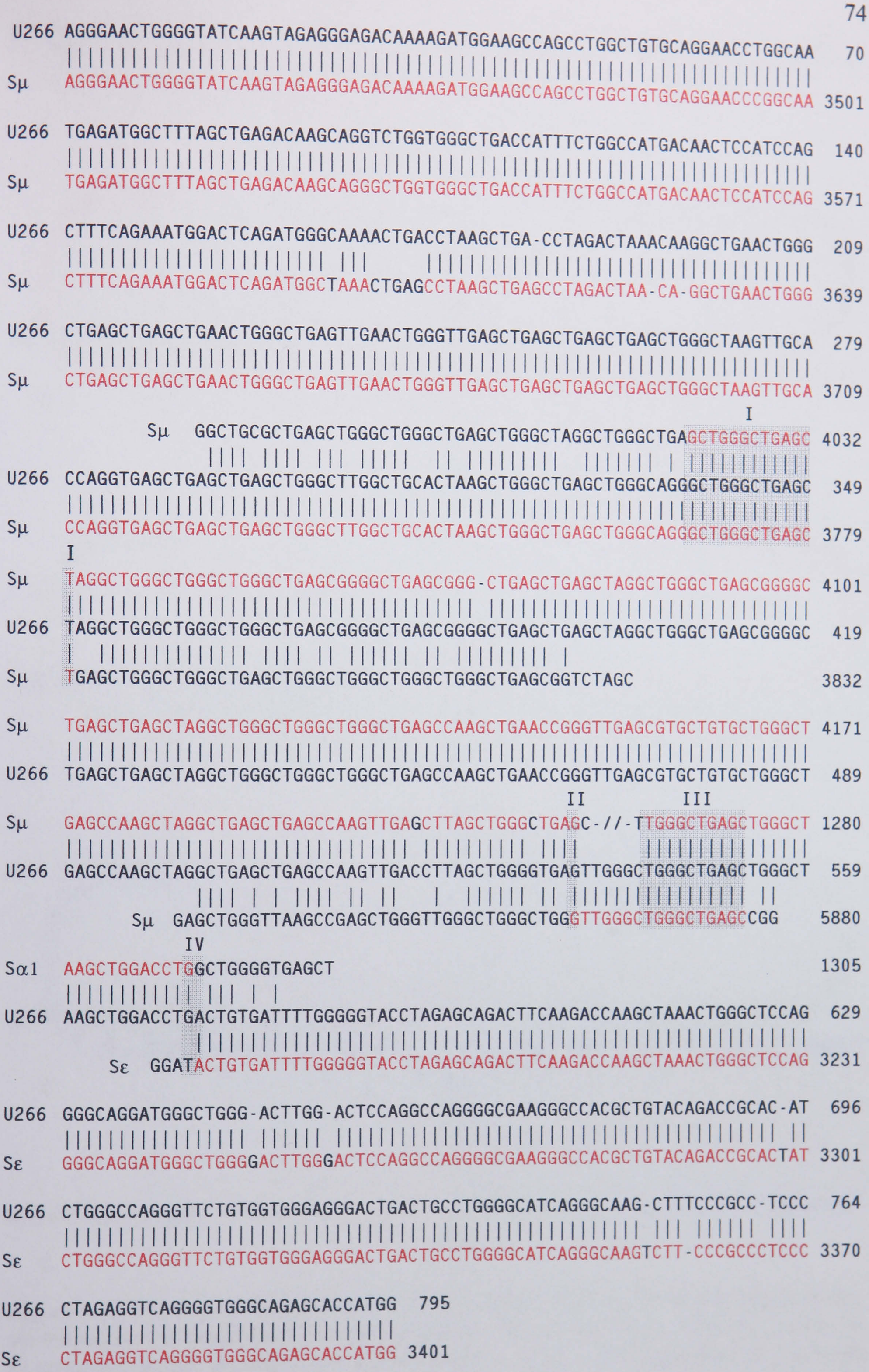
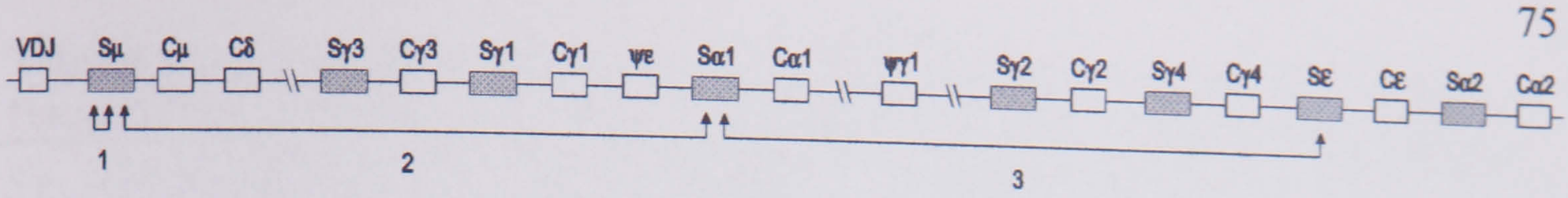
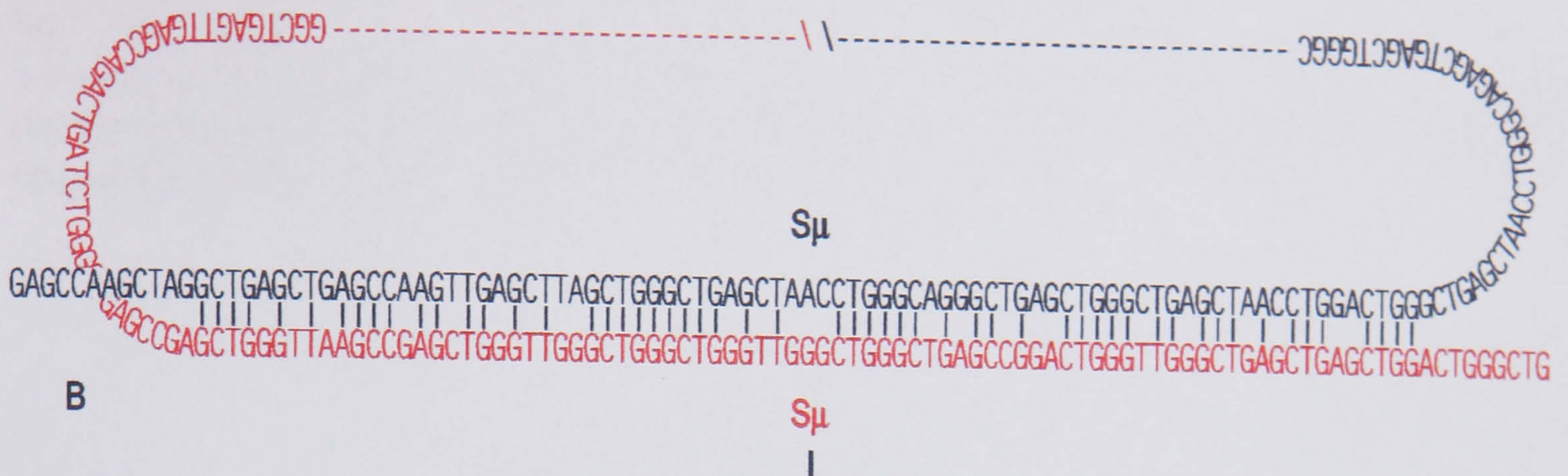


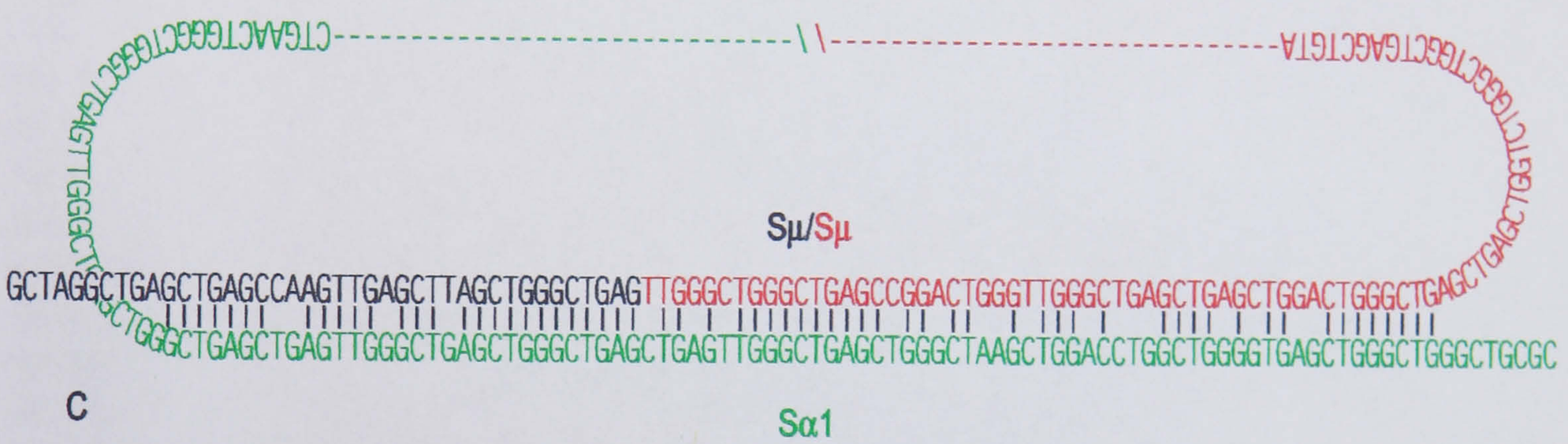
Figure 4.2. Sequence of the U266 recombination junction. The sequence of the U266 switch recombination junction is shown next to regions with identity (red) to Sμ, Sα1, and Sε. Boxes, recombination junctions.



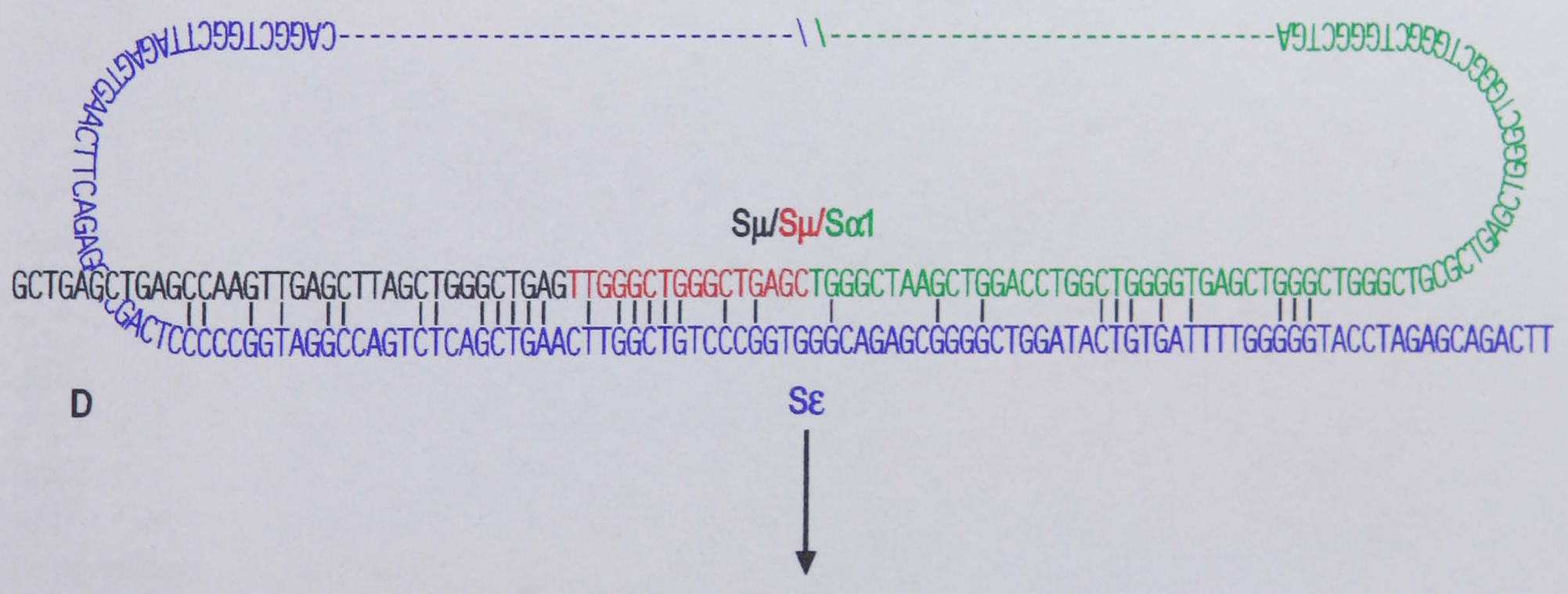
A



B



C



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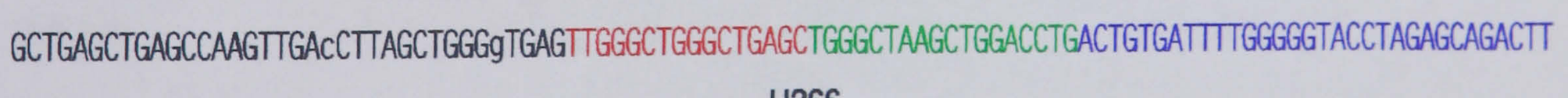


E

Figure 4.3. Proposed switch recombination events in the U266 heavy chain locus. (*A*) the location of switch recombination events in U266 on the heavy chain locus map. (*B-E*) proposed switch recombination intermediates in U266. (*1*& *B*) deletion of Sμ bases 4220-5860. (*2* & *C*) Cμ/Cα1 switching event; (*3* & *D*) Cα1/Cε Switching event; (*E*) partial sequence of U266. Vertical bars, identity between adjacent sequences. Lower case letters, sequence of unknown origin.

Table 4.1 Homology between switch regions surrounding sites of recombination.

Recombination Event	5' Switch Region[a]	3' Switch Region[a]	Homology[a]
Sμ Deletion 3781-4033	Sμ 3598-3848	Sμ 3851-4100	65%
Sμ Deletion 4220-5860	Sμ 3580-4432	Sμ 5220-6072	63%
Sμ→Sα1 Switch	Sμ 3554-7010	Sα1 591-2402	68%
Sα1→Sε Switch	Sα1 1048-1607	Sε 2929-3488	30%

[a]Homology is shown between switch region sequences (5' Switch Region 3', Switch Region) proposed to pair prior to the recombination event. The location in the published sequence, of the switch regions compared is shown.

Figure 4.4. Sequence of the switch recombination junctions. Recombination junctions from the IgE expressing plasmacytoma cell line U266, the IgE-expressing EBV lines 2C4, A5, HJS6, HJS7, the non-IgE expressing EBV line HJS102, and the fibroblast line SKS252 (Shapira et al. 1991; Mills et al. 1992; Shapira et al. 1992) (see Table 4.2 for locations). pSC1.1-4-pSC1.8-23 are DNA clones amplified by PCR from primary B cells induced with IL4 and α-CD40 (Zhang et al. 1994). Green shows regions of homology between Sμ and Sε near the switch recombination junction. Sequence was identified as originating from the Sμ region (red), Sε (blue), either Sμ or Sε (green), Sα1 (purple) unknown (lower case black). The pentamers GAGCT and GGGCT are underlined.

Table 4.2. Locations of switch recombination events[a].

Clone	Cell Type	Location Sμ	Location Sε	Event	Reference
U266	Myeloma	4219	3174	Sμ/Sε	(Shapira *et al.* 1991; Mills *et al.* 1992)
2C4	EBV line	4030	2405	Sμ/Sε	(Mills *et al.* 1992)
A5-1	EBV line	4330	2743	Sμ/Sε	(Shapira *et al.* 1991)
A5-2	EBV line	3778	2787	Sμ/Sε	(Shapira *et al.* 1991)
HJS6	EBV line	3884	2535	Sμ/Sε	(Shapira *et al.* 1991)
HJS7-2	EBV line	3592	1562	Sμ/Sε	(Shapira *et al.* 1991)
HSJ7-1	EBV line	3782	2491	Sμ/Sε	(Shapira *et al.* 1991)
pSC1.1-12	Primary B	6946	1333	Sμ/Sε	(Zhang *et al.* 1994)
pSC1.1-32	Primary B	-	1327	Sγ4/Sα1/Sε	(Zhang *et al.* 1994)
pSC1.1-35	Primary B	-	1508	Sε/Sγ4	(Zhang *et al.* 1994)
pSC1.1-4	Primary B	6689	1265	Sμ/Sε	(Zhang *et al.* 1994)
pSC1.1-40	Primary B	-	1510	Sε/Sγ4	(Zhang *et al.* 1994)
pSC1.1-7	Primary B	6819	1258	Sμ/Sε	(Zhang *et al.* 1994)
pSC1.2-65	Primary B	-	1496	Sε/Sγ4	(Zhang *et al.* 1994)
pSC1.5-1	Primary B	-	1254	Sε/Sγ4	(Zhang *et al.* 1994)
pSC1.5-8	Primary B	-	1300	Sε/Sγ4	(Zhang *et al.* 1994)
pSC1.6-5	Primary B	-	1677	Sε/Sγ4	(Zhang *et al.* 1994)
pSC1.7-1	Primary B	6952	1974	Sμ/Sε	(Zhang *et al.* 1994)
pSC1.7-12	Primary B	6783	1901	Sμ/Sε	(Zhang *et al.* 1994)
pSC1.7-2	Primary B	6951	1760	Sμ/Sε	(Zhang *et al.* 1994)
pSC1.7-22	Primary B	-	1781	Sε/Sγ4	(Zhang *et al.* 1994)
pSC1.7-4	Primary B	6659	1732	Sμ/Sε	(Zhang *et al.* 1994)
pSC1.7-56	Primary B	3849	1759	Sμ/Sγ4/Sε	(Zhang *et al.* 1994)
pSC1.7-6	Primary B	6938	1757	Sμ/Sε	(Zhang *et al.* 1994)
pSC1.7-67	Primary B	-	2395	Sε/Sγ4	(Zhang *et al.* 1994)
pSC1.8-10	Primary B	6434	1783	Sμ/Sε	(Zhang *et al.* 1994)
pSC1.8-23	Primary B	6018	1758	Sμ/Sε	(Zhang *et al.* 1994)
SKS281	Primary B	3771	2408	Sμ/Sε	(Shapira *et al.* 1992)
SKS282	Primary B	3682	2167	Sμ/Sε	(Shapira *et al.* 1992)
SKS283	Primary B	3678	2435	Sμ/Sε	(Shapira *et al.* 1992)
SKS285	Primary B	3594	2181	Sμ/Sε	(Shapira *et al.* 1992)
SKS286	Primary B	3685	3005	Sμ/Sε	(Shapira *et al.* 1992)
SKS287	Primary B	3478	1962	Sμ/Sε	(Shapira *et al.* 1992)
SKS288	Primary B	3668	2150	Sμ/Sε	(Shapira *et al.* 1992)
HJS102	EBV line	None	None	None	(Shapira *et al.* 1991)

Locations in Sμ (Appendix 5) and Sε (Appendix 6).

4.2.2 The presence of pentameric repeats in Sμ and Sε.

The pentamers GAGCT and GGGCT are present near each switch recombination junction (Fig. 4.4; Appendix 5, 6). The presence of the repeating pentamers corresponds to the location of switch recombination events in Sμ and Sε (Fig. 4.5-7). The pentamers GAGCT and GGGCT occur almost exclusively on the top strand, suggesting they may be involved in the mechanism of switching, possibly to aid in the semi-homologous pairing of switch regions prior to recombination.

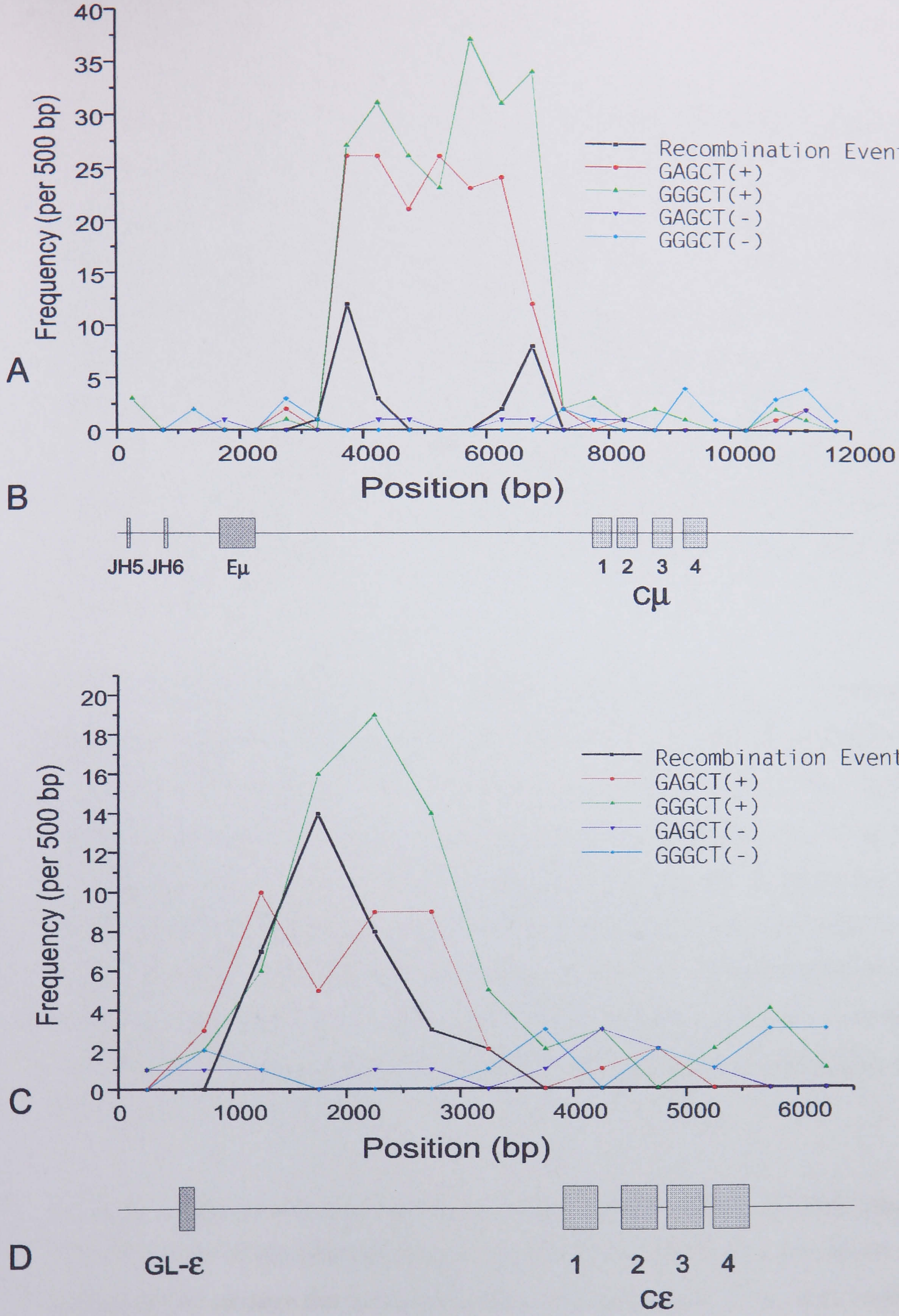


Figure 4.5. Location of pentamers and switching events in the mu and epsilon switch regions. (*A*, *C*), Frequencies of the pentamer GAGCT in the upper strand (red), and lower strand (dark blue), and the pentamer GGGCT in the upper strand (green) and lower strand (light blue) of the mu and epsilon switch regions. The frequency of switching events (recombination events, black, see Table 4.2) is also shown, compared to the relative positions in the mu and epsilon locus (*B*, *D*).

4.3 DISCUSSION

The myeloma U266 appears to be the product of a sequential switch from IgM→IgA1→IgE, leaving a 28 nucleotide Sα1 fragment between the Sμ and Sε recombination junctions. One further IgM→IgA1→IgE switching event has subsequently been published by Zhang *et* al (1994). Because IgA is not normally induced by events leading to the production of IgE, the progenitor cell for the line U266 may have initially responded to stimuli specific for switching to IgA, such as IL-10 and TGF-β (van Vlasselaer *et al.* 1992; Fayette *et al.* 1997). The U266 progenitor potentially later was exposed to Th2 cytokines, received the necessary and responded to the appropriate cell contact mediated signals for switching to IgE. The two switching events probably did not occur through a common set of stimuli since the stimuli inducing switching to IgA, such as TGF-β (Gauchat *et al.* 1990), inhibit switching to IgE.

The location of the switch recombination events were within the Sμ Sα1, and Sε regions, like subsequently published switching events (Shapira *et al.* 1991; Mills *et al.* 1992; Shapira *et al.* 1992; van der Stoep *et al.* 1994). Two deletion events occurred within Sμ. Both the deletion events, and the Sμ→α1 switch recombination event occurred in areas of significant switch region homology. The high level of homology between the switch regions near a site of recombination further supports a model where semi-homologous pairing between switch regions is important to the mechanism of switch recombination. More recent reports have demonstrated that IgM→IgE recombination events also occur at regions of homology between Sμ and Sε (Shapira *et al.* 1991; Mills *et al.* 1992; Shapira *et al.* 1992; van der Stoep *et al.* 1994).

Switching to IgE has also been reported to occur via IgG4 (Mills *et al.* 1992; Zhang *et al.* 1994). Most of the switching events via IgG4 were detected by a polymerase chain reaction (PCR) strategy that is biased to select for the presence of the Sγ4 containing switch circles (Zhang *et al.* 1994), and evidence from van der Stoep *et al* (1994) suggests that most switching events to IgE occur directly from IgM→IgE.

The repeating pentameric units GAGCT and GGGCT were present on the top strand in both the mu and epsilon switch regions. The presence of the pentamers on the top strand supports

a model that they help direct the alignment of the two switch regions prior to the recombination event by increasing the level of homology between the switch regions. The pentamers could also serve as binding sites for proteins involved in the association of the two switch regions, or for components of the recombination apparatus.

An analysis of the location of recombination events reveals that most of the recorded events occur at both borders of the areas of pentameric repeats in the mu switch region, and at the 5' border of the Sε repeats. The positioning of the recombination events at the border of the pentameric regions suggests that the structure of these regions could determine the general location of the recombination events. The role of the pentameric units could be to either aid in the positioning of recombination events by either directly interacting with the recombination apparatus, or by physically excluding recombination from the pentameric region, or by a combination of the two possibilities.

CHAPTER 5: Cloning and Characterisation of the ε-Germline Transcript.

5.1 INTRODUCTION

Prior to heavy chain switching the IgH constant region locus to which switching is directed becomes transcriptionally active (Stavnezer-Nordgren and Sirlin 1986; Lutzker and Alt 1988; Stavnezer *et al.* 1988; Berton *et al.* 1989; Esser and Radbruch 1989; Bottaro *et al.* 1994) and its transcription is referred to as germline gene transcription. Several murine and human germline transcripts have been cloned, and share structural similarities (Reviewed in Vercelli and Geha 1992; Coffman *et al.* 1993). The germline gene transcripts initiate from TATA-less promoters within 2 Kb upstream of the switch region, and proceed through short exons (germline or I_H exons) that are spliced to the first exon of the C_H gene. Germline transcripts are not translated because of multiple stop codons present in the germline exon. The region containing the germline promoter and the I_H exon is deleted during switch recombination.

One theory for the role of germline transcription in heavy chain switching is that it directs switching by modulating the accessibility of a particular switch region to a common recombinase. Thus, the specificity of switching may be directed via the regulation of germline gene transcription. *In vitro* experiments also suggest that germline transcripts form tri-molecular complexes with the switch region and may be involved in the association of the two switch regions prior to recombination (Reaban *et al.* 1994; Fujieda *et al.* 1996).

Germline transcription is a required event for switch recombination to occur. The importance of germline transcription in isotype switching is shown by gene knock-out experiments. Deletion of the Iγ1 or Iγ2b exons and their promoters resulted in the inhibition of class switching to the corresponding genes (Jung *et al.* 1993; Zhang *et al.* 1993). The loss of switching after deletion of the germline exon and promoter suggests that transcription in the switch region is necessary to target the appropriate switch region for recombination and switching. Transcription itself, although necessary, is not sufficient for switch recombination. An $E\mu/V_H$ promoter cassette, containing no exon sequences, cannot restore normal levels of switch recombination when inserted in place of the ε-germline gene promoter and exon (Bottaro *et al.* 1994). Furthermore, conservation of

the germline exon 3' splice donor sequence is required in addition to transcription for normal levels of switch recombination, suggesting that a correctly spliced germline transcript is required (Lorenz *et al.* 1995).

With the essential role of germline transcription in switch recombination well established, it is therefore important to understand how the induction of germline transcription is regulated. Different cytokines specifically activate transcription at the appropriate germline promoter. In particular, IL-4 has been shown to induce ε germline transcripts in murine B cells (Rothman *et al.* 1990; Severinson *et al.* 1990; Rothman *et al.* 1991).

The work in this chapter describes the cloning of the human germline gene transcript for the heavy chain gene of IgE. Additionally the ε germline gene transcription start sites were determined in primary B cells and B cell lines. A comparison of the genomic sequence between three individual people has revealed a polymorphism in the germline exon.

5.2 RESULTS

5.2.1 Structure of the ε-germline gene transcript.

To clone the cDNA for the ε-germline transcript, polymerase chain reaction (PCR) primers were designed near regions of homology between the human switch region sequence and the mouse germline-ε (GL-ε) exon (Fig. 5.1). Homology between the mouse GL-ε exon and a 538 base segment upstream of the human epsilon switch region was 67%, and potentially contained the human GL-ε exon.

RNA was isolated from PBMC incubated with IL-4 for 5 days. Second strand cDNA was synthesised (see Materials and Methods 2.16) and used as a template for PCR using primers specific for the putative germline exon and Cε4, yielding a 1497 base product (Figs. 5.2, 5.3; Table 5.1). To clone the 5' end of the cDNA, circular PCR was performed on cDNA that was cleaved at a HincII site in the 3' end of Cε1 and circularised to its 5' end. Primers specific for the germline exon, and the 3' end of εCH1 were used to amplify a product across the 5' end of the germline exon and the ligated ends of the cDNA circle. The PCR products were inserted into Bluescript and sequenced (Fig. 5.4; Table 5.2).

```
Human   1 GGATCCCCGGCTGCAGGACAGTGACCTGGGAGTGAGTACAAGGTGAGGCC 50
          |||  |  |  ||||  |  ||  |  |  ||  ||  |||||  |  ||  |||  |||||
Mouse 214 GGACCTCAAGCTGAAAGATAATCACTTGTGAGTGGGCACCAGGGAAGGCC 263

Human  51 AC...CACTCAGGGTGCCAGCTCCAAGCGGGTCACAGGGACGAGGGCTGC 97
          ||    |  |||  |  ||||||||||||     |  |||||  ||  ||||  |||
Mouse 264 ACTGTCCCTC.GCATGCCAGCTCCAAAGCTGATACAGGAACTAGGG.TGC 311

Human  98 GGCCATCAGGAGGCCCTGCA..CACACATCTGGGACACGCGCCCCGAGGG 145
            |  ||||  ||||||||||||     ||  ||||||              ||  |  |
Mouse 312 CTCTATCA.GAGGCCCTGCAATGTCATATCTGG.........CCCACAG 350

Human 146 CCAGTTCACCTCAGTGCGCC.TCATTCTCCTGCACAAAAGCGCCCCCATC 194
            |  ||||  ||  ||||  ||  |  |  |  |  |  ||  |    |  ||  ||
Mouse 351 GCTGTTCCTCTTTGTGCACCATTAATAACTTACAAAGTGACAGCCACA.. 398

Human 195 CTTTCTTCACAAGGCTTTCGTGGAAGCAGAGGCGTCGATGCCCAGTACCC 244
            ||  |  ||||||  |  |  |  ||||  |  |||  |  ||
Mouse 399 ..CTCCCCTGAAGGCTGCCAAAGGAACAGAAAAAGCAATGGCGAGGGTGT 446

Human 245 TCTCCCTTTCCCAGGCAACGGGACCCCAAGTTTGCTGAC.TGGGACCACC 293
            |||  |  ||||  |  ||  ||||  ||  |  |  |||  |  |
Mouse 447 AGTCCTGCCTCAGGGCAGTGACACTCCAAAGGGGCAGGCATGGTGACTGC 496

Human 294 AAGC.CACGCATGCGTCAAGAGTAGAGTCCGGGACCTAGGC.AGGGGCCC 341
            |  ||  |||  ||||  ||||  |  |  |  ||  ||  |||  ||  |||  |  ||
Mouse 497 ACGCACACACATG...CAAGGCTTTAATACGAGAGCTATGCAAGGAGACC 543

Human 342 TGGCGTTGGGCCTGAGAGAGAAGAGAACCTCCCCAGCACT....CGGTGT 387
            |||  |  |  |  ||||  |||||  |||  |  |  |  |||||
Mouse 544 TGGGATCAGACGATGGAGAATAGAGAGCCTTGACCAGAGTGTGCAGGTGT 593

                                                    H
Human 388 GCATCGGTAGTGAACCAGCCTCACCTGA.CCCCCGCTGTTGCTCAATCGA 436
            |  ||  |||  ||  ||||||||||||  ||||  ||||  ||  |  ||  |
Mouse 594 GTCTC.CTAG.AAAGAGGCCTCACCTGAGACCCCACTGTGCCTTAGTCAA 641

                  G
Human 437 CTTCCCAAGAACAGAGAGAAAAGGGAACTTCCAGGGCGGCCCGGGCCTCC 486
            |||  ||||||||||||  |||  ||||||||||||  |||  ||  ||  ||
Mouse 642 CTT.CCAAGAACAGAATCAAAAGGGAACTTCCAAGGCTGCTAAGG...CC 687

Human 487 TGGGGTTCCCACCCCATTTTTAGCTGAAAGCACTGAGGCAGAGCTCCCCC 536
            ||||||||||||||  ||||||||||  ||||||||||||||  ||||
Mouse 688 GGGGGTTCCCACCCCACTTTTAGCTGAGGGCACTGAGGCAGAGCGGCCCC 737

Human 537 TA 538
            ||
Mouse 738 TA 739
```

Figure 5.1. Homology between the sequences surrounding the mouse GL-ε exon and the corresponding area upstream of the human switch region. The human (Mills et al. 1990) and mouse (Gerondakis 1990) sequences are compared. The mouse GL-ε exon is shown in red. Arrows represent the position and orientation of the oligonucleotides G and H used for cloning the germline transcript.

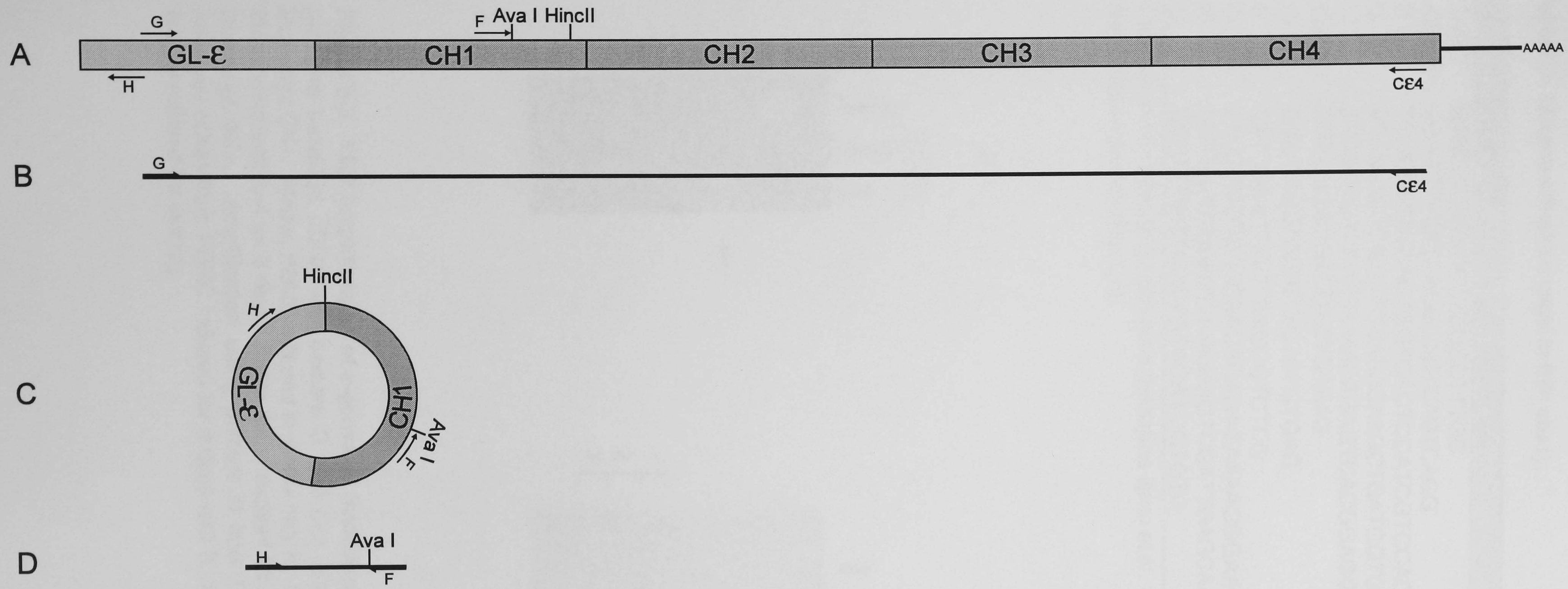


Figure 5.2. PCR cloning of the ε-germline transcript. (A) The GLε cDNA. (B) The PCR product resulting from amplification with primer G in the germline exon (Table 5.1), and Cε4 in the constant region. (C) The cDNA circle formed by ligation of the 5' end of the germline transcript to the HincII restriction site near the end of ε-CH1. (D) The PCR product from amplification of the cDNA circle with primer H in the germline exon and primer F in Cε1 (Table 5.1). Arrows, oligonucleotide primers.

Table 5.1 Oligonucleotides used in this study.

Oligo	Sequence	Location
Actin F	GAGCACAGAGCCTCGCCTTTGC	267-288[a]
Actin R	GGATCTTCATGAGGTAGTCAGTCAGG	2242-2217[a]
B	TCTAGAGCTCACTAGTCACTCCATCGTCCACAGACTG	534-554[b]
C	ATCGATCTCGAGTCGACGCAAGCTGATGGTGGCATAGTGAC	458-481[b]
C2	ATCGATCTCGAGTCGACAGAGTCACGGAGGTGGCATTG	325-347[b]
Cε2.3	CGTCGCAGGACGACTGTAAG	613-633[b]
Cε4	CGCTCGCTGGACGGTCTGTGAG	1408-1429[b]
F	CATCAGCTTGCTGACCGTCTCG	470-491[b]
G	CGAATTCCCAAGAACAGAGAGAAAAGGGAACTTCCAAGG	36-69[b]
H	ACTAGTCTAGACTTGGGAAGTCGATTGAGCAAC	22-43[b]
Q	CCATTTGGTGCCTGTGGATGGTGTG	227-251[b]

Location in the [a]human β-actin gene (Nakajima-Iijima et al. 1985), or the [b]ε-germline gene transcript sequence (Fig.5.4).

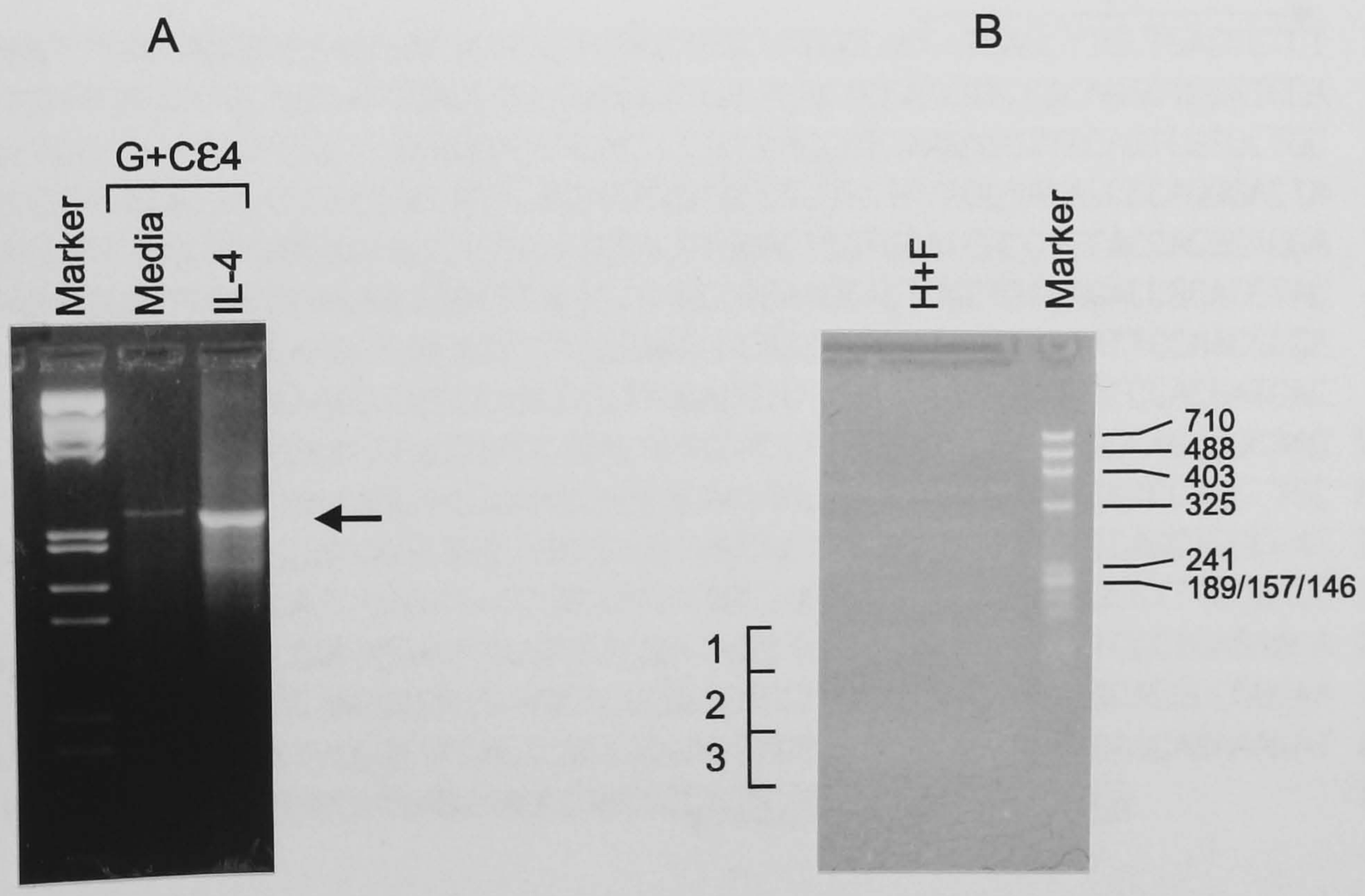
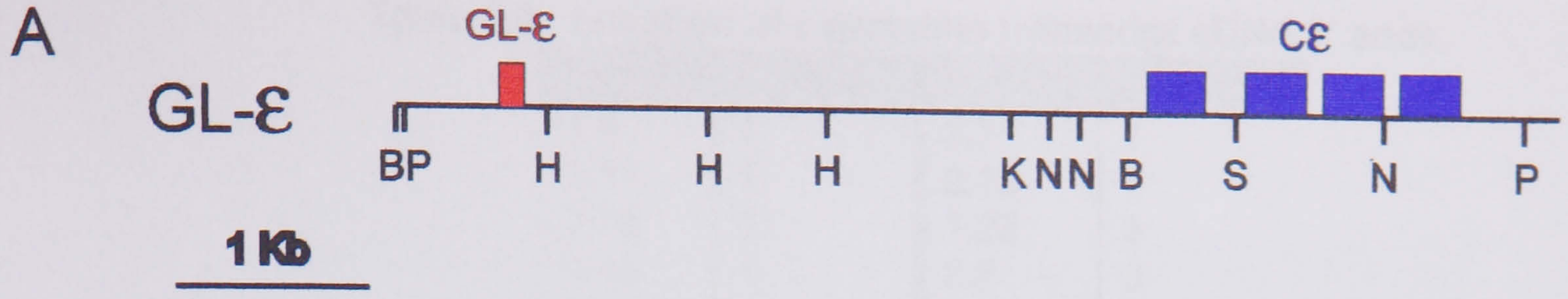


Figure 5.3. PCR amplification of ε-germline transcript cDNA. (*A*) Amplification of ε-germline transcript cDNA with primers G and Cε4 (Table 5.1). Markers, *Hpa*II cut bluescript SK+. Media, PBMC cultured in media only for 3 days. IL-4, PBMC cultured in the presence of IL-4 for 3 days. Arrow, band excised for cloning. (*B*) Markers, *Hpa*II cut bluescript SK+. Amplification using primers H and F, of the 5' end of ε-germline transcript cDNA from PBMC induced for 3 days with IL-4 . Arrows indicate 3 regions of the gel excised for cloning.



B

```
                                                                    G
GAGCCTCACCTGACCCCCGCTGTTGCTCAATCGACTTCCCAAGAACAGAGAGAAAAGGGAACTTCCAGGG    70
                                                  H
CGGCCCGGGCCTCCGGGGGTTCCCACCCCATTTTTAGCTGAAAGCACTGAGGCAGAGCTCCCCCTACCCA   140
GGCTCCACTGCCCGGCACAGAAATAACAACCACGGTTACTGATCATCTGGGAGCTGTCCAGGAACCCGAC   210
AGGGAGCCGGACGAGCCACACCATCCACAGGCACCAAATGGACGACCCGGCGCTTCAGCCTCCACACAGA   280
                                  Q
GCCCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCCAATGCCACCTCCGTGACTCTGGG   350
CTGCCTGGCCACGGGCTACTTCCCGGAGCCGGTGATGGTGACCTGCGACACAGGCTCCCTCAACGGGACA   420
                                                                F
ACTATGACCTTACCAGCCACCACCCTCACGCTCTCTGGTCACTATGCCACCATCAGCTTGCTGACCGTCT   490
CGGGTGCGTGGGCCAAGCAGATGTTCACCTGCCGTGTGGCACACACTCCATCGTCCACAGACTGGGTCGA   560
CAACAAAACCTTCAGCGTCTGCTCCAGGGACTTCACCCCGCCCACCGTGAAGATCTTACAGTCGTCCTGC   630
GACGGCGGCGGGCACTTCCCCCCGACCATCCAGCTCCTGTGCCTCGTCTCTGGGTACACCCCAGGGACTA   700
TCAACATCACCTGGCTGGAGGACGGGCAGGTCATGGACGTGGACTTGTCCACCGCCTCTACCACGCAGGA   770
GGGTGAGCTGGCCTCCACACAAAGCGAGCTCACCCTCAGCCAGAAGCACTGGCTGTCAGACCGCACCTAC   840
ACCTGCCAGGTCACCTATCAAGGTCACACCTTTGAGGACAGCACCAAGAAGTGTGCAGATTCCAACCCGA   910
GAGGGGTGAGCGCCTACCTAAGCCGGCCCAGCCCGTTCGACCTGTTCATCCGCAAGTCGCCCACGATCAC   980
CTGTCTGGTGGTGGACCTGGCACCCAGCAAGGGGACCGTGAACCTGACCTGGTCCCGGGCCAGTGGGAAG  1050
CCTGTGAACCACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCACGTTAACCGTCACGTCCACCCTGC  1120
CGGTGGGCACCCGAGACTGGATCGAGGGGGAGACCTACCAGTGCAGGGTGACCCACCCCCACCTGCCCAG  1190
GGCCCTCATGCGGTCCACGACCAAGACCAGCGGCCCGCGTGCTGCCCCGGAAGTCTATGCGTTTGCGACG  1260
CCGGAGTGGCCGGGGAGCCGGGACAAGCGCACCCTCGCCTGCCTGATCCAGAACTTCATGCCTGAGGACA  1330
TCTCGGTGCAGTGGCTGCACAACGAGGTGCAGCTCCCGGACGCCCGGCACAGCACGACGCAGCCCCGCAA  1400
GACCAAGGGCTCCGGCTTCTTCGTCTTCAGCCGCCTGGAGGTGACCAGGGCCGAATGGGAGCAGAAAGAT  1470
GAGTTCATCTGCCGTGCAGTCCATGAGGCAGCGAGCCCCTCACAGACCGTCCAGCGAGCG            1429
                                                  Cε4
```

Figure 5.4. Sequence of the ε germline transcript. (*A*) Map of the epsilon locus in germline configuration. (*B*) Sequence of the germline exon (red), and of the constant region exons (blue). Binding sites of the PCR primers (arrows) used to clone the 5'end (H, F), and the 3' end (G, Cε4) of the germline transcript. The stop codons (underlined), and the only ATG (black), are indicated.

Table 5.2. Location of ε-germline transcript cDNA 5' ends.

Clone[a]	Location[b]	Clone[a]	Location[b]
1.4	1	2.7	1
1.11	1	2.13	1
1.12	1	1.22	3
1.13	1	2.5	3
1.14	1	2.6	3
1.15	1	2.11	3
1.16	1	2.12	3
1.17	1	2.3	19
1.18	1	2.4	20
1.19	1	2.8	20
1.21	1	2.9	20
2.1	1	2.10	21/22

[a]5' ends of cDNAs cloned by circular PCR.
[b]Location in the germline transcript sequence (Fig.5.4)

The Cε exons of the germline transcript showed 100% identity to the published sequence (Flanagan and Rabbitts 1982). The sequence of the GL-ε exon was 98% similar to the published sequence upstream of Sε (Fig. 5.5; Mills *et al.* 1990), and situated about 500 bases upstream of the switch region (Fig. 4.5; Appendix 6). Each of the 4 clones sequenced were identical except for position 224, which had either an A or G and potentially represented a polymorphism.

To confirm that a polymorphism exists at position 224, and determine the correct sequence for the GL-ε exon, we cloned genomic DNA for this region by PCR (Fig. 5.6; see Materials and Methods 2.18). Primers were chosen from the published genomic sequence 380 bp upstream of the germline exon, and 17 bp downstream of the exon. Nineteen clones were analysed from the DNA of 3 individuals (Table 5.3). The sequences agreed at all positions except for either a G or A at nt 604 of the genomic sequence. Of the three individuals analysed, one was homozygous for the A allele, and two heterozygous. The sequence of the cDNA clones agreed 100% with either the A or G allele of the genomic DNA sequence.

Similar to the germline transcripts previously reported for μ, γ2β, and ε (Lennon and Perry 1985; Lutzker and Alt 1988; Rothman *et al.* 1990), an in-frame AUG is not present in the germline exon. The only ATG found in the ε-germline exon is translationally out of frame with the constant region (Fig.5.4). In addition, stop codons are present in each reading frame in the germline exon, consistent with the observation that germline transcripts are not translated.

The intronic sequence at the intron/exon border of the ε-germline exon (Figs. 5.4, 5.6) obeys the GT.....intron.....AG rule (for splicing review, see Padgett *et al.* 1986).

```
38A  CAGCCTCACCTGACCCCCGCTGTTGCTCAATCGACTTCCCAAGAACAGAG 452
     ||||||||||||||||||||||||||||||||||||||||||||||||||
cDNA GAGCCTCACCTGACCCCCGCTGTTGCTCAATCGACTTCCCAAGAACAGAG 50

38A  AGAAAAGGGAACTTCCAGGGCGGCCCGGGCCTCCTGGGGTTCCCACCCCA 502
     |||||||||||||||||||||||||||||||| ||||||||||||||||||
cDNA AGAAAAGGGAACTTCCAGGGCGGCCCGGGCCTCCGGGGGTTCCCACCCCA 100

38A  TTTTTAGCTGAAAGCACTGAGGCAGAGCTCCCCCTACCCAGGCTCCACTG 552
     ||||||||||||||||||||||||||||||||||||||||||||||||||
cDNA TTTTTAGCTGAAAGCACTGAGGCAGAGCTCCCCCTACCCAGGCTCCACTG 150

38A  CCCGGCACAGAAATAACAACCACGGTTACTGATCATCTGGGAGCTGTCCA 602
     ||||||||||||||||||||||||||||||||||||||||||||||||||
cDNA CCCGGCACAGAAATAACAACCACGGTTACTGATCATCTGGGAGCTGTCCA 200

38A  GGAACCCGACA..GAGCCGGA.GGGCCACA.CATCCACAGGCACCAAATG 648
     |||||||||||  ||||||||  | ||||||  ||||||||||||||||||
cDNA GGAACCCGACAGGGAGCCGGACG[A/G]GCCACACCATCCACAGGCACCAAATG 250

38A  GACGACCCGGCGCTTC 664
     ||||||||||||||||
cDNA GACGACCCGGCGCTTC 266
```

Figure 5.5. Identity between the GL-ε and published Sε sequences. The GL-ε exon sequence (cDNA) is compared with the published genomic Sε sequence from λ clone 38A (Mills et al. 1990).

```
CCTGGGAGTGAGTACAAGGTGAGGCCACCACTCAGGGTGCCAGCTCCAAGCGGGTCACAGGGACGAGGGC  70
TGCGGCCATCAGGAGGCCCTGCACACACATCTGGGACACGCGCCCCGAGGGCCAGTTCACCTCAGTGCGC 140
CTCATTCTCCTGCACAAAAGCGCCCCCATCCTTTCTTCACAAGGCTTTCGTGGAAGCAGAGGCGTCGATG 210
CCCAGTACCCTCTCCCTTTCCCAGGCAACGGGACCCCAAGTTTGCTGACTGGGACCACCAAGCCACGCAT 280
GCGTCAAGAGTGAGAGTCCGGGACCTAGGCAGGGGCCCTGGGGTTGGGCCTGAGAGAGAAGAGAACCTCC 350
CCCAGCACTCGGTGTGCATCGGTAGTGAAGGAGCCTCACCTGACCCCCGCTGTTGCTCAATCGACTTCCC 420
AAGAACAGAGAGAAAAGGGAACTTCCAGGGCGGCCCGGGCCTCCGGGGGTTCCCACCCCATTTTTAGCTG 490
AAAGCACTGAGGCAGAGCTCCCCCTACCCAGGCTCCACTGCCCGGCACAGAAATAACAACCACGGTTACT 560
GATCATCTGGGAGCTGTCCAGGAACCCGACAGGGAGCCGGACG[A/G]GCCACACCATCCACAGGCACCAAATG 630
GACGACCCGGCGCTTCAGGTATCCCAGCCCACC 663
```

Figure 5.6. Genomic sequence surrounding the GL-ε exon. Red, GL-ε exon; PCR primers, horizontal arrows.

Table 5.3. Occurrence of the polymorphism at position 604 in the germline exon.

Donor	#A alleles[a]	#G alleles[a]
1	6	0
3	4	2
6	2	5

[a]Number of PCR generated clones with the A or G allele sequence from the genomic DNA of three donors (1, 3, and 6).

5.2.2 ε-germline transcript expression.

Expression of the ε-germline gene transcript was investigated in cultures of PBMC and in the B lymphoma line Namalwa (Middleton *et al.* 1991). PBMC were induced with IL-4 over a 13 day period and assayed at 12 hours, 1, 2, 3, 5, 7, 9, 11, and 13 days for both secreted IgE by enzyme-linked immunoabsorbent assay (ELISA), and the ε-germline gene transcript by PCR (Fig. 5.7). Secreted IgE became detectable at day 5 in PBMC, and reached a maximum at day 9 of culture with IL-4. Germline transcript levels were detectable by RT-PCR as early as 12 hours after IL-4 addition, and reached a maximum at day 3 after IL-4 addition, supporting the model that the induction of germline transcription precedes switching. ε-GLT were also detectable, at lower levels, without added IL-4 during a shorter time period between 3 and 9 days of culture. The expression of germline transcripts without added IL-4 is presumably due to production of type 2 cytokines by T cells activated during or prior to the initiation of the culture. The B cell line Namalwa also induced the expression of the ε-GLT after treatment with IL-4 over an 8 day period, with the expression again undetectable 22 days after IL-4 addition, showing that an IL-4 stimulus is required for the maintenance of germline transcription. After 22 days of culture, all of the IL-4 activity was presumably degraded. Germline transcription was also detected in an IL-4 induced EBV B cell line (A5), in Tonsil cells induced with IL-4, but not with the IgE secreting plasmacytoma U266 (Nilsson *et al.* 1970). The lack of ε-GLT expression in the cell line U266 agrees with other reports (Gauchat *et al.* 1990), and is presumably due to the fact that heavy chain recombination occurred on both chromosomes, deleting the ε-germline exon.

5.2.3 Location of ε-germline transcript start sites.

To find the transcription start site, S1 protection was performed on RNA isolated from IL-4 treated PBMC and primer extension was performed on the EBV-negative Burkitt lymphoma B cell lines, BJAB (Clements *et al.* 1975) and BL-2 (Gauchat *et al.* 1992). S1 protection involves hybridisation of a single stranded DNA probe, ^{32}P-labelled at its 5' end, and complementary to a mRNA of interest. The probe is hybridised to total RNA and the single stranded ends of the hybrid are digested with S1 nuclease, leaving a probe corresponding in length to the 5' end of the transcript. The digested hybrids are run on a denaturing

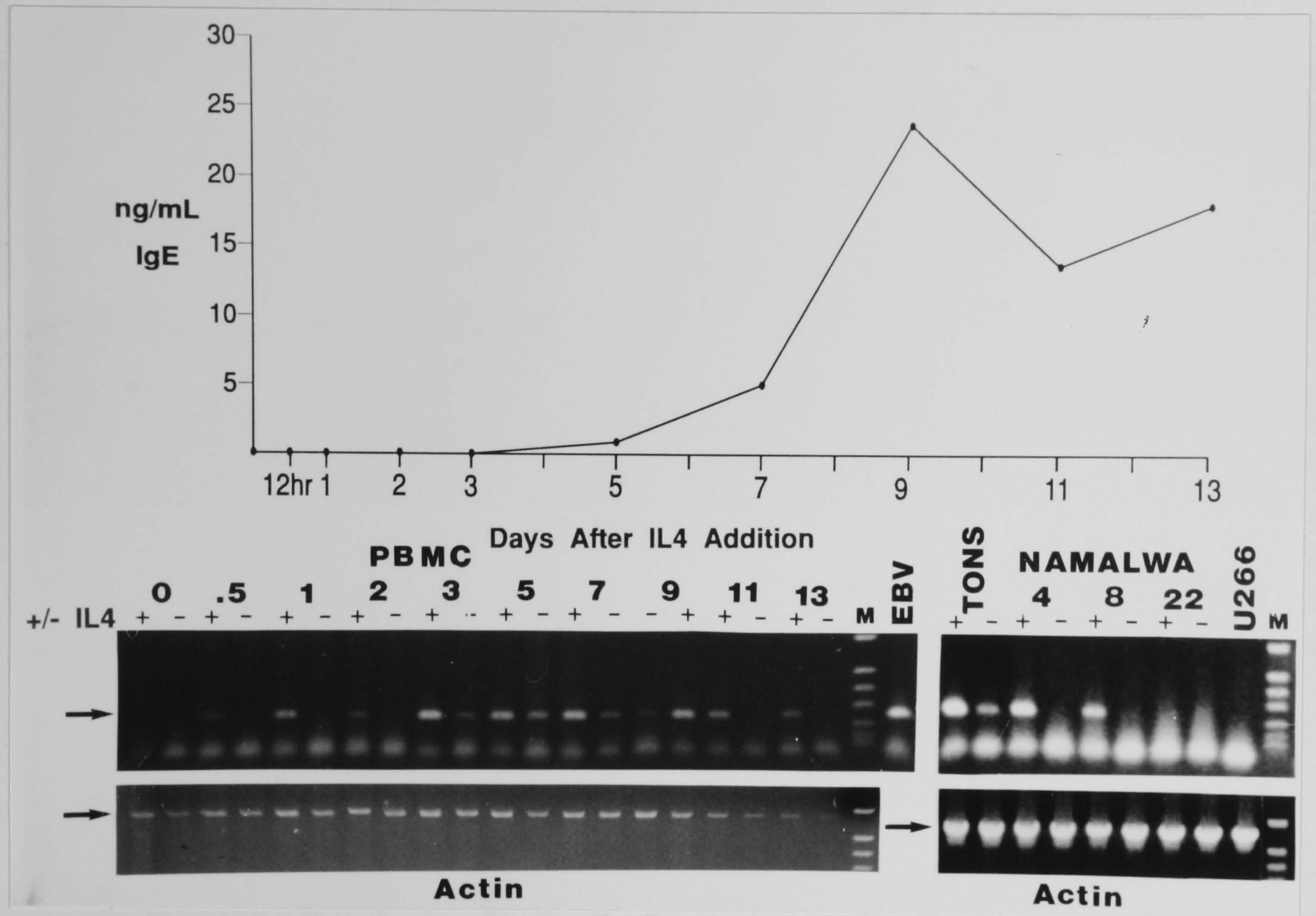


Figure 5.7. Expression of the ε-germline transcript. (*A*) IgE levels secreted by PBMC induced with IL-4 for 12 hr, 1, 2, 3, 5, 7, 9, 11, and 13 days, measured by ELISA. (*B*), (*C*) Parallel cultures assayed for GLε and β-actin levels, respectively, by RT-PCR. EBV, the Epstein-Barr virus infected IgE producing cell line A5, induced with IL-4. M, The molecular weight marker Bluescript cut with Hpa II. (*D*), (*E*) RT-PCR analysis of tonsil PBMC (TONS), cultured with and without IL-4, the EBV positive Burkitt lymphoma cell line Namalwa, induced with and without IL-4 for 4, 8, and 22 days, and the IgE producing myeloma U266. The GLε and β-actin oligonucleotide PCR primers used are listed in Table 5.1.

acrylamide gel and sized against a DNA sequencing ladder. Primer extension is performed by hybridising a short fragment of complementary DNA to the RNA and extending the fragment by a DNA synthesis reaction off the 5' end of the RNA with reverse transcriptase. The primer extension products are also sized on a denaturing acrylamide gel. BJAB was induced with IL-4 alone, whereas BL-2 was induced with both IL-4 and α–CD40. A probe from (392 to -251) was used for S1 analysis of PBMC RNA (Fig.5.9). Signals equivalent between the uninduced and IL-4 induced lanes were considered to be due to background, and disregarded. In PBMC, multiple start sites were found by S1 analysis at positions -35, -21, 53, 108, 119, 132, 141, 166, 170, and 190 (Figs. 5.9, 5.10). Consistent with the presence of multiple start sites, no TATA box is found in the upstream sequences. Promoters with multiple start sites commonly lack a TATA element (Benoist and Chambon 1981). The previously cloned germline transcripts for μ, γ2b, and ε also possessed multiple start sites and lacked a TATA box (Lennon and Perry 1985; Lutzker and Alt 1988; Rothman *et al.* 1990). Primer extension was initiated from an oligonucleotide probe at positions 251 to 227. Multiple start sites were also found in the B cell lines BJAB and BL-2 by primer extension at positions 21-25, 30, 81, 102, 106, 110, 140, 141, and 157 (Figs. 5.8, 5.10). Some of the transcription start sites in PBMC generally agree with those in the cell lines BJAB and BL-2, namely positions 140/141, 53/57, and 102/108. The differences between the start sites determined in PBMC and the cell lines could be due to the influence of the activation or differentiation state each cell line is arrested in on the presence of various transcription factors that affect transcriptional initiation. Differences in the levels of some of the transcription factors in the cell lines could cause changes in the position of initiation events from those present in PBMC. Gauchat *et al* (1990) also reported multiple transcription start sites in IL-4 stimulated PBMC for the GL-ε transcript (Fig. 5.10, green arrows). The start sites determined for PBMC in this chapter agreed within 3 bp to the start sites reported by Gauchat *et al* (1990) . One site determined for PBMC in this chapter at base 119 was not found in Gauchat *et al*, though this was a minor start site not confirmed in the cell lines BJAB and BL-2. Because a shorter probe was used by Gauchat *et al*, containing a 5' terminus at base 77 of the GL-ε promoter sequence, sites 5' of base 77 could not be compared. The 3' terminus of the transcription start site region reported in this chapter, agreed exactly with Gauchat *et al*.

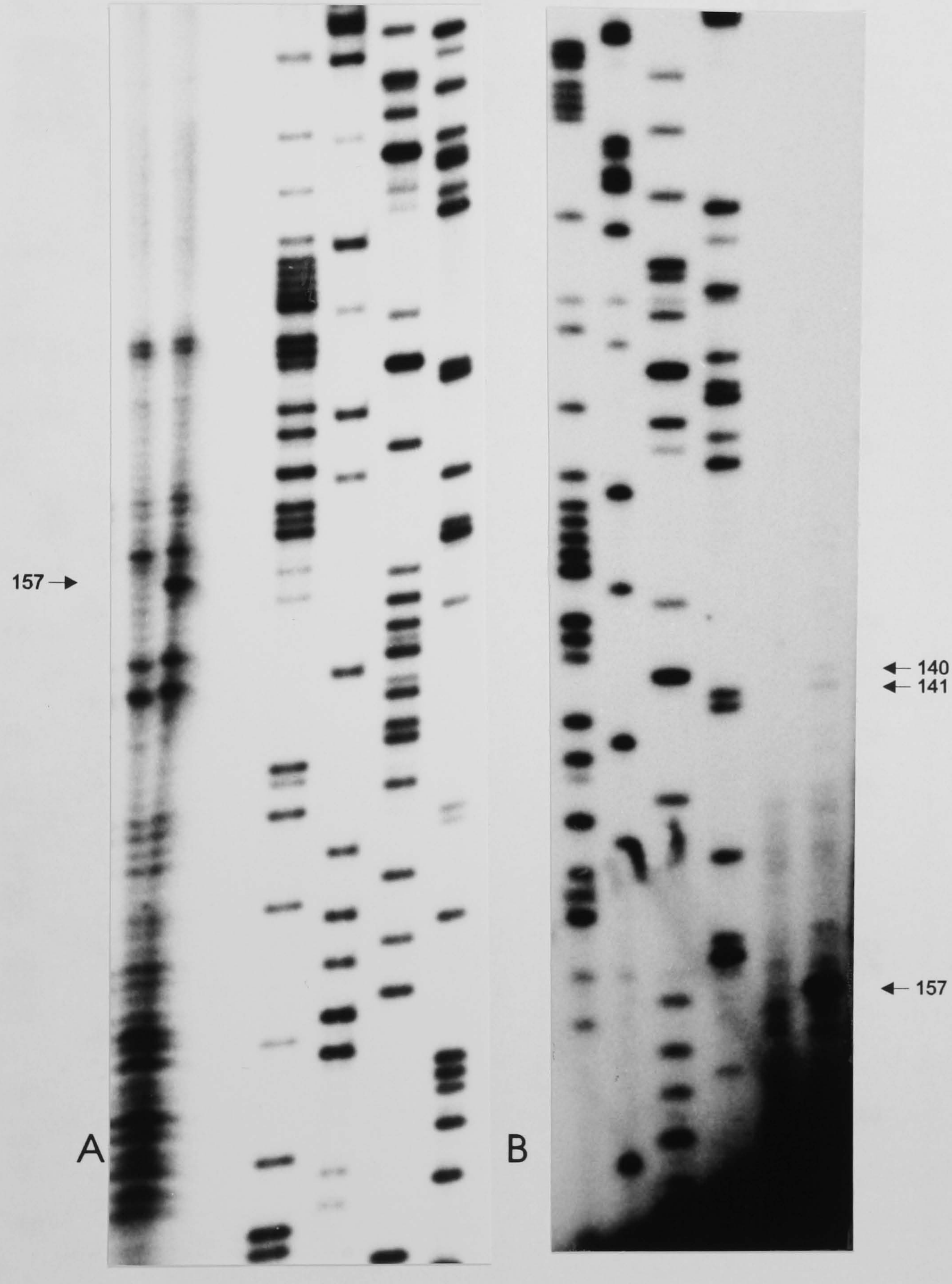


Figure 5.8. Primer extension analysis of ε-germline transcript start sites in the B-lymphoblastic line BJAB. (*A*) Sequence ladder (G, A, T, C), using oligo Q as a sequencing primer. (BJAB, BJAB+IL-4), primer extension analysis of BJAB cells with, and without added IL-4, using oligo Q as a primer. The arrow indicates the start site induced in BJAB cells with IL-4 at position 157. (*B*) (G, A, T, C), Sequence ladder using oligo STFR as a primer. (BJAB, BJAB+IL4), Primer extension analysis of BJAB with, and without added IL-4, using oligo STFR as a primer. The arrows indicate the start sites induced in BJAB cells with IL-4 at positions 140, 141, and 157.

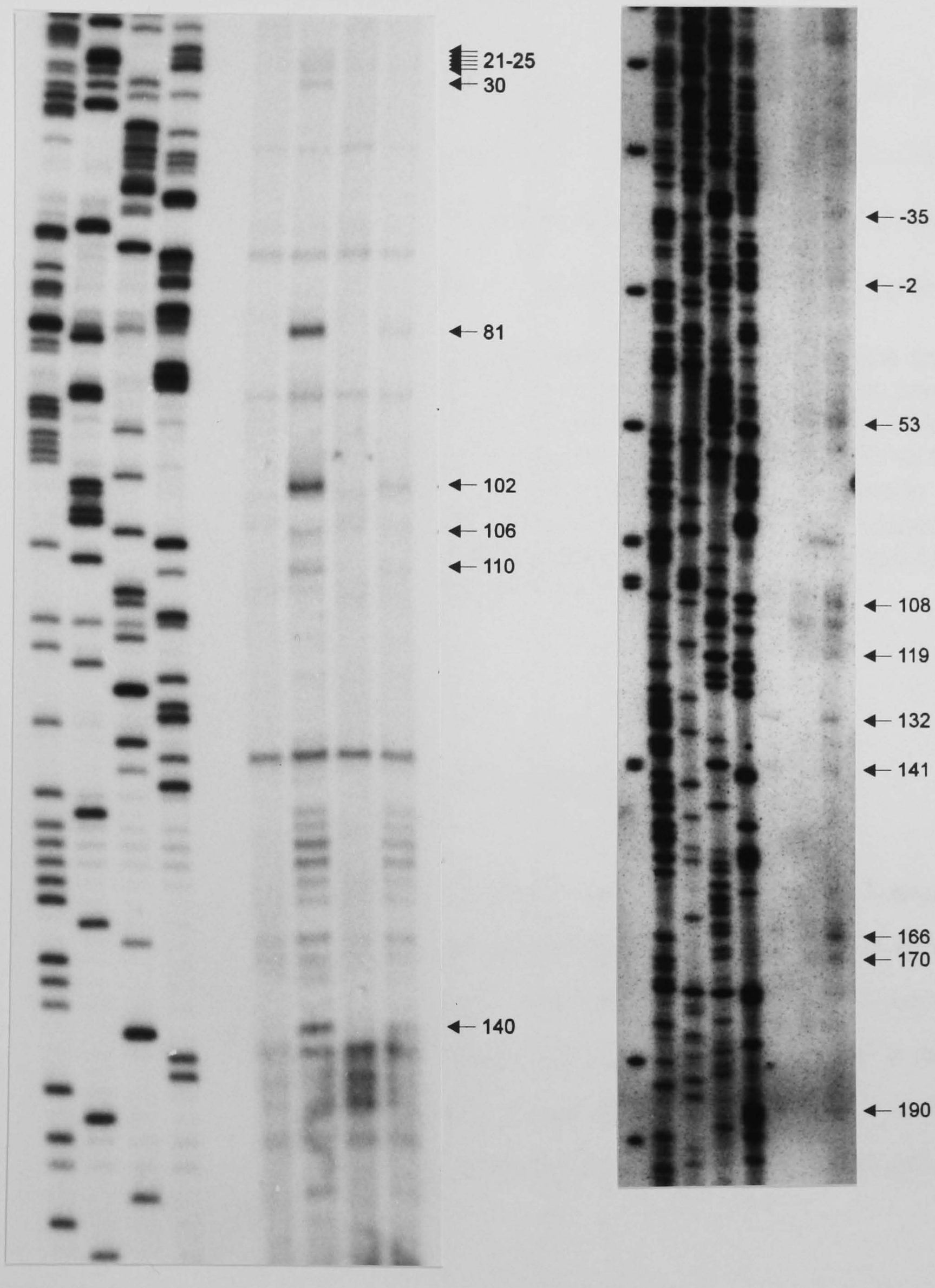


Figure 5.9. S1 nuclease mapping of the ε-germline transcript start sites in the B-lymphoblastic lines BJAB and BL-2, and PBMC. (*A*) Sequence ladder (G, A, T, C), using oligo STFR as a sequencing primer., Primer extension analysis of BJAB cells with and without added IL-4 (BJAB, BJAB+IL-4), using oligo STFR as a primer. Primer extension analysis of BL-2 cells with, and without added IL-4 and αCD40 antibody (BL-2, BL-2+IL-4+αCD40), using oligo STFR as a primer. The arrows indicate the start sites induced in BJAB and BL-2 cells with IL-4 at positions 21-25, 30, 81, 102, 106, 110, and 140. (*B*) (Marker), HpaII cut pBluescript SK+, sequence ladder (G, A, T, C), using oligo Q as a sequencing primer. S1 protection analysis of PBMC with, and without added IL-4 (PBMC, PBMC+IL4). The arrows indicate the start sites induced in PBMC with IL-4 at positions -35, -2, 53, 108, 119, 132, 141, 166, 170, and 190.

Figure 5.10. Location of transcription start sites for the germline transcript. Red sequence, germline sequence, black, genomic sequence. Black arrows, start sites determined by S1 nuclease protection with RNA from IL4 induced PBMC. Blue arrows, start site determined by primer extension using the cell lines BJAB and BL-2 induced with IL4 and IL4+α-CD40, respectively. Q, the oligonucleotide used for primer extension, and to construct the S1 probe (see materials and methods). Red arrows, location of the cDNA 5' ends detected by PCR (Table 5.2, Fig.5.4). Green arrows, start sites determined by Gauchat *et al* (1990).

5.3 DISCUSSION

The germline transcript for IgE was cloned from IL-4 treated PBMC. Transcription initiated about 500 bases upstream of Sε at multiple sites in a region of about 220 bp in primary cells and in the B cell lines BJAB and BL-2. The location of the all of the multiple initiation sites presented in Gauchat *et al* (1990) agreed with the start sites presented in this chapter. No TATA box is present immediately upstream of the germline exon, consistent with the observation that promoters which possess multiple initiation sites commonly lack a TATA element.

The ε-germline gene transcript was expressed as early as 12 hours after IL-4 addition, and reached a maximum at day 3 after IL-4 addition, supporting the model that the induction of germline transcription precedes switching. ε-GLT were also detectable without added IL-4 during a shorter time period between 3 and 9 days of culture. The expression of germline transcripts without added IL-4 is presumably due to production of type 2 cytokines by T cells activated during or prior to the initiation of the culture. ε-

germline gene transcripts were also expressed after IL-4 induction in the B cell line Namalwa over an 8 day period, with the expression again undetectable 22 days after IL-4 addition, showing that a continuous IL-4 stimulus is required for germline transcription.

A polymorphism exists at base 224 of the cDNA sequence with either an A or G at this position. Three individuals were analysed for the presence of this polymorphism. Two individuals were heterozygous for the polymorphism and one was homozygous for the A allele. No linkage is known between the polymorphism and atopy. The polymorphism is 34 bases downstream of the 3'-most transcription initiation site and is unlikely to affect the regulation of transcription (Figs. 5.6, 6.1B). Because the germline transcript may physically interact with the recombination apparatus, or form a tri-molecular complex with the switch regions, a polymorphism in the germline exon could destabilise these interactions, and affect the level of switching.

Like all other germline transcripts characterised, the ε germline exon contains stop codons in each translational reading frame and does not possess an AUG codon in frame with the constant region, consistent with the conclusion that no translation of germline transcripts occurs.

The sequence of the ε-germline exon agrees with Gauchat *et al* (1990) except at positions 467 and 641 (Fig 5.6), containing a C and T instead of a G and T, respectively at these positions, in disagreement with both Mills et al (1990) and the sequence presented here. Also, the designation of the germline exon in Gauchat *et al* excluded one base at the 3' end of the exon which forms the first base of the epsilon constant region. The expression kinetics for the ε-GLT in PBMC, presented in Gauchat *et al*, agree with the results presented here except that the maximum for transcription levels was later, at about 9 days after IL-4 addition, though transcription controls were not used in Gauchat *et al*, so accurate judgement of their transcription levels was not possible.

CHAPTER 6: Role of BSAP/PAX-5 in the Regulation of ε–Germline Transcription.

6.1 INTRODUCTION

Isotype switching results from a DNA recombination event that juxtaposes different downstream CH genes to the expressed VDJ gene segment, thus changing the effector function but not the specificity of the antibody molecule. Isotype switching is not a random event, but is directed by cytokines in conjunction with the regulation of B cell proliferation and differentiation (Coffman *et al.* 1993). Cytokine-dependent induction of isotype switching to a particular CH gene almost invariably correlates with the transcriptional activation of the same gene in germline configuration. Several murine and human germline transcripts have been cloned, and share structural similarities (for review see Vercelli and Geha 1992; Coffman *et al.* 1993). Germline transcripts initiate from TATA-less promoters a few kilobases upstream of the switch region, and proceed through short exons (germline or IH exons) that are spliced to the first exon of the CH gene. The region containing the germline promoter and the IH exon is deleted during switch recombination.

Germline transcription is thought to direct switching by modulating the accessibility of a particular switch region to a common recombinase. The importance of germline transcription in the regulation of isotype switching has been demonstrated by gene knock-out experiments. Deletion of the Iγ1 (Jung *et al.* 1993) or Iγ2b (Zhang *et al.* 1993) exons and their promoter resulted in the inhibition of class switching to the corresponding genes, suggesting that transcription in the switch region is necessary to target the appropriate switch region for recombination and switching. Therefore, it is important to understand how germline transcription is regulated.

Different cytokines specifically activate transcription at the appropriate germline promoter. In particular, IL-4 has been demonstrated to induce ε-germline transcripts in murine (Rothman *et al.* 1990; Severinson *et al.* 1990; Rothman *et al.* 1991), and human (Gauchat *et al.* 1990; Jabara *et al.* 1990) B cells. IL-4-dependent ε-germline transcription in B cells is strongly up-regulated upon CD40 engagement (Jabara *et al.* 1990; Shapira *et al.* 1992). This transcriptional effect of CD40 may be critical for switching (Warren and Berton 1995), because optimal levels of transcription through the switch region may be required to target recombination.

Several cis-acting elements have been characterised which regulate IL-4-induced ε-germline transcription. A highly conserved DNA region 5' to the major initiation sites for murine germline-ε RNA has been demonstrated to contain an IL-4-responsive element that binds three transcription factors: a member of the Signal Transducer and Activator of Transcription (STAT) family, Stat6, one or more members of the CCAAT/enhancer-binding protein (C/EBP) family, and Nuclear factor-kappa B (NF-κB)/p50 (Delphin and Stavnezer 1995, see also Fig. 6.2). Mutation of any of the binding sites for these factors abolished or reduced the IL-4 inducibility of the murine ε-promoter (Delphin and Stavnezer 1995). A binding site for Stat6 has also been identified in the human ε-germline promoter (Kohler and Rieber 1993; Albrecht *et al.* 1994; Fenghao *et al.* 1995).

A region in the murine ε-germline promoter 3' to the Stat6 element binds a constitutively expressed transcription factor, B cell lineage-specific activator protein (BSAP, Pax-5) (Liao *et al.* 1994). BSAP belongs to the Pax gene family of homeodomain transcription factors, and is the mammalian homologue of the sea urchin tissue-specific transcription activator protein, a regulator of late histone genes. BSAP is expressed in pre-B and mature B lymphocytes, but not in terminally differentiated plasma cells (Adams *et al.* 1992). BSAP knock-out mice exhibit a complete block of early B cell differentiation at the pro-preBI stage (Urbanek *et al.* 1994), and are significantly reduced in V-DJ recombination (Nutt *et al.* 1997). Binding sites for BSAP have been identified in the promoter of several B cell-specific genes, such as CD19 (Kozmik *et al.* 1992), λ5, VpreB1 (Okabe *et al.* 1992), XBP 1 (Reimold *et al.* 1996), the tyrosine kinase *blk* (Zwollo and Desiderio 1994), and the germline gene exons and switch regions of the IgH genes μ, γ1, γ2, ε, α (Waters *et al.* 1989; Liao *et al.* 1992; Xu *et al.* 1992; Liao *et al.* 1994). BSAP has also been demonstrated to positively regulate the CD19 gene, the B-cell receptor component Ig-alpha (mb-1) and the transcription factors N-myc and LEF-1 (Kozmik *et al.* 1992; Nutt *et al.* 1998). BSAP negatively regulates the XBP 1 promoter (Reimold *et al.* 1996), and the gene coding for the cell surface protein PD-1 (Nutt *et al.* 1998). Interestingly, BSAP positively regulates the Ig 3' α enhancer HS4 (Michaelson *et al.* 1996), but has a negative effect on the Ig 3' α enhancer HS1,2 (Singh and Birshtein 1993; Neurath *et al.* 1994) Negative regulation of the Ig 3' α enhancer HS1,2 seems to be due to the ability of BSAP to suppress binding of the Ets family member NF-αP, a protein that positively controls enhancer activity and heavy chain transcription (Neurath

et al. 1995). BSAP interacts with the POU domain of octamer binding proteins to mediate its repression of the 3' enhancer (Singh and Birshtein 1996). In addition to the suggested interaction with NF-αP and octamer binding proteins, BSAP has been demonstrated to participate in other protein-protein interactions, including its facilitation of the binding of proteins of the Ets proto-oncogene family to the early B-cell-specific mb-1 promoter (Fitzsimmons *et al.* 1996). Further evidence for the role of BSAP in recruiting other transcription factors to regulatory regions was demonstrated by the reconstitution of mb-1 and LEF-1 activity in BSAP deficient pre-BI cells by the BSAP paired domain polypeptide lacking any transactivation function (Nutt *et al.* 1998).

BSAP has also been demonstrated to be involved in the control of cell proliferation in mouse B cells (Wakatsuki *et al.* 1994). Furthermore, ≈2% of non-Hodgkin lymphomas are generated by translocation of the BSAP gene near the Eμ enhancer (Busslinger *et al.* 1996). BSAP may be important for switching not only through the regulation of genes in the immunoglobulin locus, but also through the control of cellular proliferation. Through the use of DNA synthesis inhibitors, proliferation has been shown to be important for γ1-germline transcription and switching (Lundgren *et al.* 1995). In addition, Ets family members have been implicated in the cell cycle specific control of γ1-germline transcription.

Of the factors known to bind the ε-germline transcript promoter in mice and humans, BSAP represents the only B cell specific factor. As the only B cell specific factor, BSAP may be responsible for the cell type specificity of ε-germline transcription. Conflicting reports exist though, about the role of BSAP in the regulation of ε-germline transcription in murine B cells. Deletion of the BSAP binding site abrogated induction of ε-germline promoter activity in murine B cells stimulated with IL-4 + LPS (Liao *et al.* 1994). Activity was restored by the introduction of a BSAP binding site from the sea urchin histone gene H2A-2.2 (Liao *et al.* 1994). In contrast, deletions in the BSAP binding region were unable to affect the IL-4-dependent inducibility of the promoter in the absence of LPS (Delphin and Stavnezer 1995). In this chapter, the role of BSAP in the transcriptional regulation of the ε-germline promoter in human B cells was investigated. BSAP was found to bind the human ε-germline promoter, and to be essential for both the IL-4-dependent induction and CD40-mediated up-regulation of human ε-germline transcription.

6.2 RESULTS

6.2.1 Characterisation of cell lines to be used for transcription factor analysis.

The human B cell lines BJAB and BL-2 were chosen as candidates for studying the regulation of ε-germline transcription. BJAB and BL-2 have not undergone a heavy chain switching event and are EBNA-negative. EBV-encoded proteins provide B cells with signals that trigger IgE switching (Thyphronitis *et al.* 1989; Jabara *et al.* 1990), through a pathway shared at least in part with the CD40 signaling route (Cheng *et al.* 1995; Mosialos *et al.* 1995). EBV positive cell lines were therefore avoided because of the potential difficulties in the interpretation of experimental results. BJAB and BL-2 were assayed by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for the expression of ε-germline transcripts upon IL-4 stimulation. Figure 6.1 illustrates that BJAB and BL-2 cells expressed ε-germline transcripts following a 48 hr incubation with IL-4 (100 U/mL). In the cell line BL-2, ε-germline transcript levels were significantly lower than BJAB in the presence of IL-4 alone, and were normally not detectable. In the presence of α-CD40 (5 μg/mL) and IL-4, ε-germline transcript levels in BL-2 were enhanced at least 5 fold over IL-4 alone. ε-germline transcription was not induced by α–CD40 mAb alone in the cell line BL-2 (Fig. 6.1, right panel). Germline transcript levels were quantified by densitometery and normalised to levels of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to control for errors in loading.

Next, EMSA was used to identify B cell specific proteins that bind to the germline transcript promoter. To test that the EMSA was functioning in our system, an IL-4 induced signal corresponding to STAT6 (Fenghao *et al.* 1995) was assayed from IL-4 induced BJAB cells. A probe that spans the STAT6 site (nucleotides 36-78 in Fig. 6.2B) was used for EMSA analysis. As early as 5 hours after addition of IL-4, a signal was substantially enhanced (Fig. 6.3) in nuclear extracts from cells induced with IL-4 and IL-4/α-CD40. The IL-4 induced complex was present up to 48 hours after IL-4 addition. The presence of an IL-4 induced complex binding to the STAT6 site strongly suggests that the EMSA correctly detected nuclear proteins that bind to the ε–germline promoter.

Table 6.1 Oligonucleotides used in this study.

Name	Primer Pair (upper strand, lower strand)	Size[a]	T°[b]	Reference/*Accession*
ε-GLT	GACGGGCCACACCATCCACAGGCACCAAATGGACGAC	409	65°C	Fig. 5.4 nt 220-256
	CAGGACGACTGTAAGATCTTCACG			Fig. 5.4 nt 606-629
GAPDH	GGGAAGGTGAAGGTCGGAGTC	438	58°C	*M33197* nt 64-84
	CTGATGATCTTGAGGCTGTTG			*M33197* nt 483-503
104/139	TTAGCTGAAAGCACTGAGGCAGAG	36	57°C	Fig. 5.4 nt 104-127
	GGGTAGGGGGAGCTCT			Fig. 5.4 nt 124-139
104/191	TTAGCTGAAAGCACTGAGGCAGAG	89	60°C	Fig. 5.4 nt 104-127
	CCCAGATGATCAGTAACCGTG			Fig. 5.4 nt 171-191
36/266	CGAATTCCCAAGAACAGAGAGAAAAGGGAACTTCCAAGG	229	67°C	Fig. 5.4 nt 36-69
	CCATTTGGTGCCTGTGGATGGTGTG			Fig. 5.4 nt 227-266
H2A2.2	TGTGACGCAGCGGTGGGTGACGAC	24		(Barberis et al. 1989)
	GTCGTCACCCACCGCTGCGTCACA			
NF-HB	GATCAGAATTGTGAAGCGTGACCA	24		(Liao et al. 1992)
	TGGTCACGCTTCACAATTCTGATC			
COUP	TCGACTCTATGGTGTCAAAGGTCAAACTTCTGAC	34		(Wang et al. 1987)
	CTCAGAAGTTTGACCTTTGACACCATAGAGTCGA			
TRE	CGATCGTGCGGTCACGTCCCTGATCTTACCTTTCCA	36		(Orchard et al. 1990)
	TGGAAAGGTAAGATCAGGGACGTGACCGCACGATCG			
BSAP1XMut	TTAGCTGAAAGCACTGAGGAAGAG	89	50°C	Fig. 5.4 nt 104-127
	CCCAGATGATCAGTAACCGTG			Fig. 5.4 nt 171-191
BSAP2XMut	TTAGCTGAAAGCACTGAGTAAGAG	89	50°C	Fig. 5.4 nt 104-127
	CCCAGATGATCAGTAACCGTG			Fig. 5.4 nt 171-191
ε–GLTREP	CCTGGGAGTGAGTACAAGGTGAG	663	64°C	*X56797* nt 25-47
	GGTGGGCTGGGATACCTGAAG			*X56797* nt 667-687
REP1XMUT	CTAGCAAATAGGCTGTCCC	379	50°C	Promega
RVprimer3				
	GGGAGCTCTTCCTCAG			Fig. 5.4 nt 117-132
BSAP Del1	CACCCCATTTTTAGCTCCCAGGCTCCACTGC	274	47°C	Fig. 6.2B nt 94-151
	GCAGTTGCTCTCCAGCGGTTC			Promega GL4
BSAP Del2	CTAGCAAATAGGCTGTCCC	379	50°C	Promega
RVprimer3				
	AGCTAAAAATGGGGTG			Fig. 6.2B nt 94-109

[a]Size of PCR product, or ds oligonucleotide. [b]Annealing temperature used for PCR amplification.

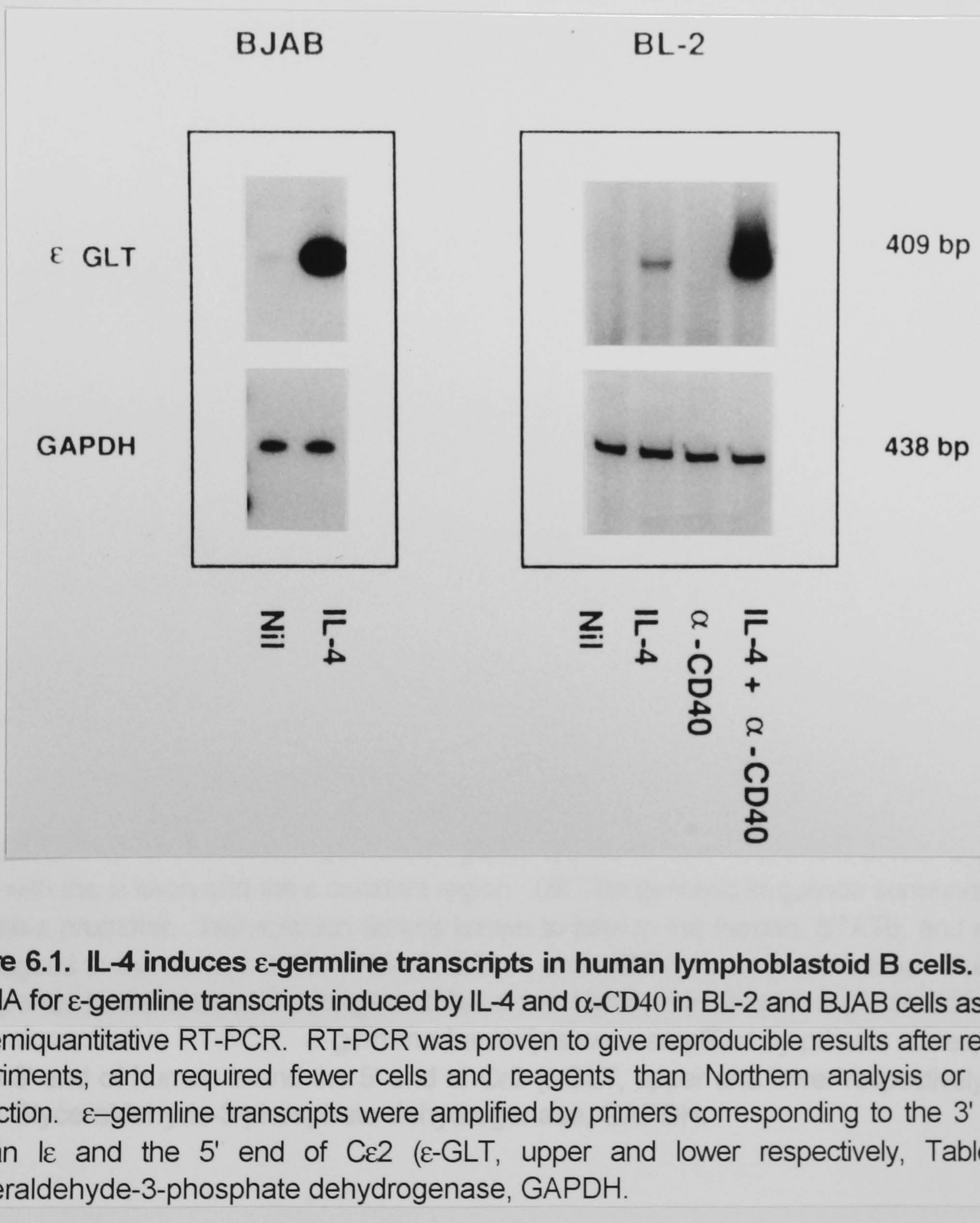
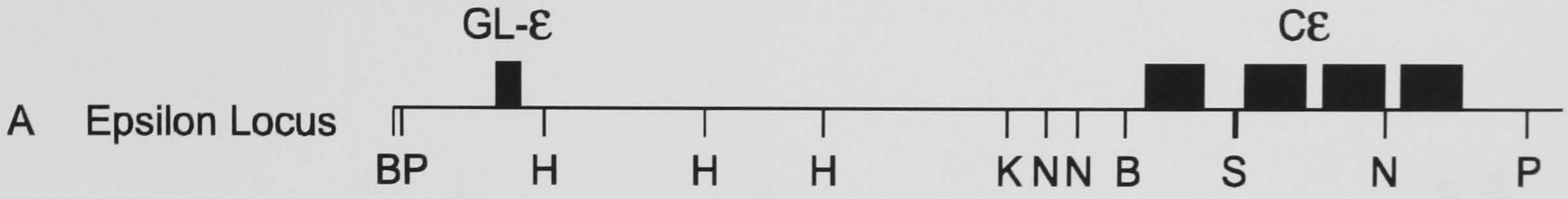


Figure 6.1. IL-4 induces ε-germline transcripts in human lymphoblastoid B cells. Levels of RNA for ε-germline transcripts induced by IL-4 and α-CD40 in BL-2 and BJAB cells assessed by semiquantitative RT-PCR. RT-PCR was proven to give reproducible results after repeated experiments, and required fewer cells and reagents than Northern analysis or RNase protection. ε–germline transcripts were amplified by primers corresponding to the 3' end of human Iε and the 5' end of Cε2 (ε-GLT, upper and lower respectively, Table 6.1). Glyceraldehyde-3-phosphate dehydrogenase, GAPDH.



B

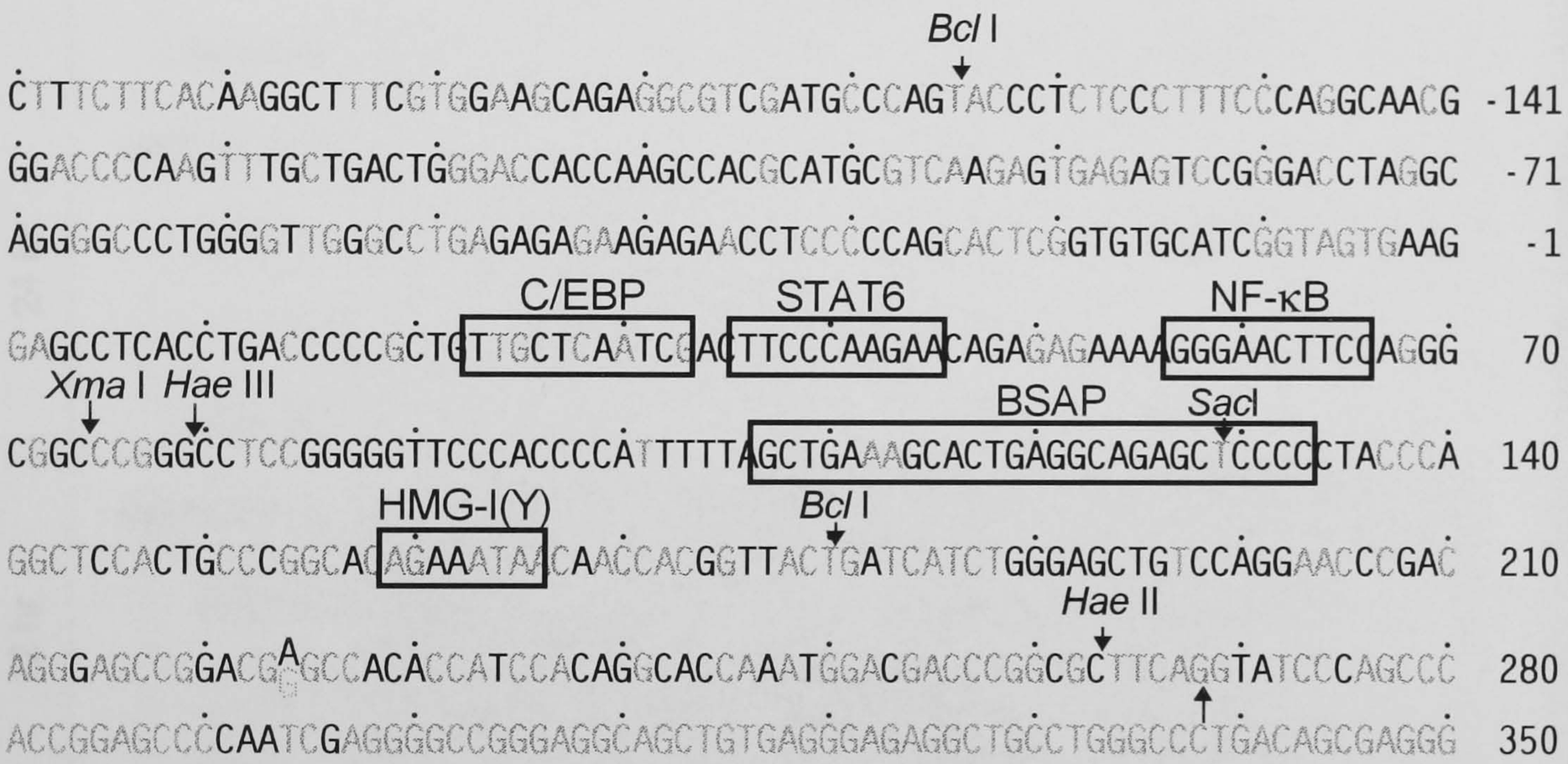


Figure 6.2. Map of the human germline-ε gene. (*A*) Restriction map of the unrearranged Cε locus, with the Iε exon and the ε constant region. (*B*) The genomic sequence surrounding the germline-ε promoter. Transcription factors known to bind in the human, STAT6, and regions homologous to the mouse C/EBP, NF-κB, BSAP, and HMG-I(Y), and , are boxed. Position 1 corresponds to the start of the longest cDNA clone obtained in chapter 5 (nucleotide 405 in Genbank accession no. X56797). Black and grey sequence indicate bases homologous and not homologous, respectively to the mouse Iε region. The end of the Iε exon is marked by an Arrow. B, Bam HI; P, Pst I; H, Hind III, K, Kpn I; N, Nco I; S, Sal I.

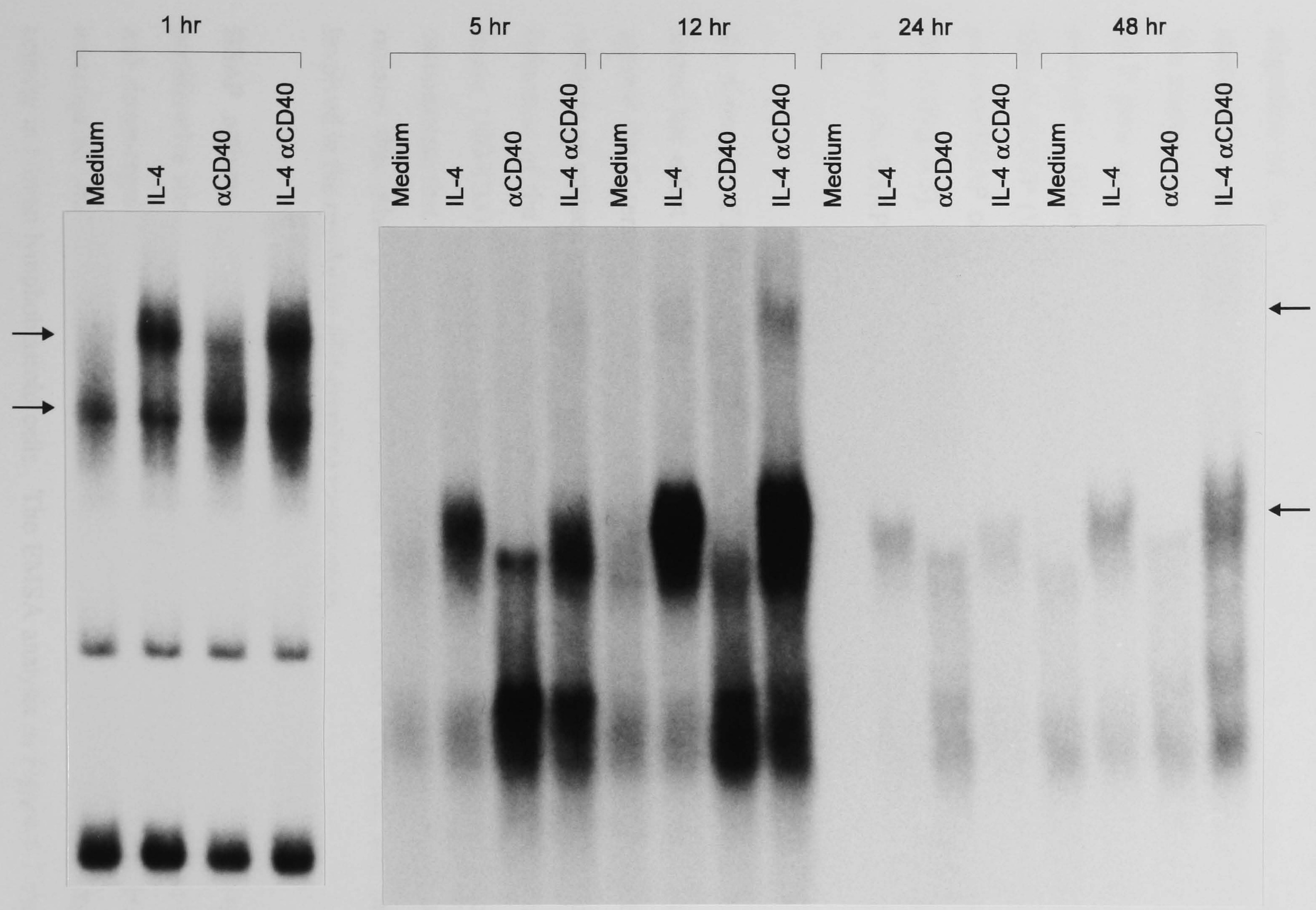


Figure 6.3. A complex is enhanced by IL-4 and IL-4/α-CD40 in BJAB nuclear extracts. EMSA with a probe from nucleotides 36-78, and extracts from BJAB cells cultured medium, IL-4, α-CD40, or both for (*A*) 1 and (*B*) 5, 12, 24 and 48 hours. (The arrows show the position of two complexes enhanced by IL-4 and IL-4/α-CD40.

6.2.2 BSAP/PAX-5 binds to the ε-GLT promoter.

In order to assess whether BSAP binds to the human ε-germline promoter, EMSA analysis was performed using a probe (nucleotides 80-184 in Figure 6.2B) that spans the region homologous to the murine BSAP binding site. Figure 6.4 demonstrates that nuclear extracts from unstimulated BJAB cells contain a protein that retards the migration of the BSAP probe. The formation of the DNA-protein complex was specifically competed by an oligonucleotide containing a BSAP site located upstream of the murine Sγ2a region, previously named B-lineage-specific nuclear factor that binds to Ig H gene segments (NF-HB), and by a BSAP site in the sea urchin histone H2A-2.2 promoter. Oligonucleotides containing the binding sites for the unrelated transcription factors COUP (Wang *et al.* 1987) and TRE (Orchard *et al.* 1990) did not compete the putative BSAP complex. The putative BSAP complex also was found in the B cell line BL-2 (Fig. 6.5). Consistent with the B cell specificity of BSAP expression (Adams *et al.* 1992), the BSAP complex was undetectable in nuclear extracts from Jurkat T cells (Fig. 6.5).

To determine if BSAP was present in the complex that binds to the BSAP probe, we tested the effect of α–BSAP antisera in EMSA. Figure 6.6 shows that an antiserum against the C-terminal region of BSAP (aa 189-391) supershifted the BSAP complex, whereas an antiserum against the DNA binding domain of BSAP (aa 17-145) blocked the formation of the complex. In contrast, an antiserum against an unrelated transcription factor (HOXB3) had no affect on the binding of BSAP. These results combine to demonstrate that BSAP binds to the human ε-germline promoter *in vitro*. The possibility remains that other proteins may participate in the EMSA complex, and may also be involved in the regulation of ε-germline transcription.

BSAP activity in splenic murine B cells has been shown to be up-regulated by proliferative stimuli (mitogens, cross-linking of sIgD or CD40) (Wakatsuki *et al.* 1994), and down-regulated by OX40 ligand cross-linking (Stuber *et al.* 1995). We therefore investigated whether stimulation with IL-4 and/or α–CD40 mAb could modulate BSAP activity in human lymphoblastoid cells. The EMSA analysis in Figure 6.7 indicates that IL-4 and α-CD40 stimuli do not cause significant changes in BSAP levels in nuclear extracts from BL-2 cells.

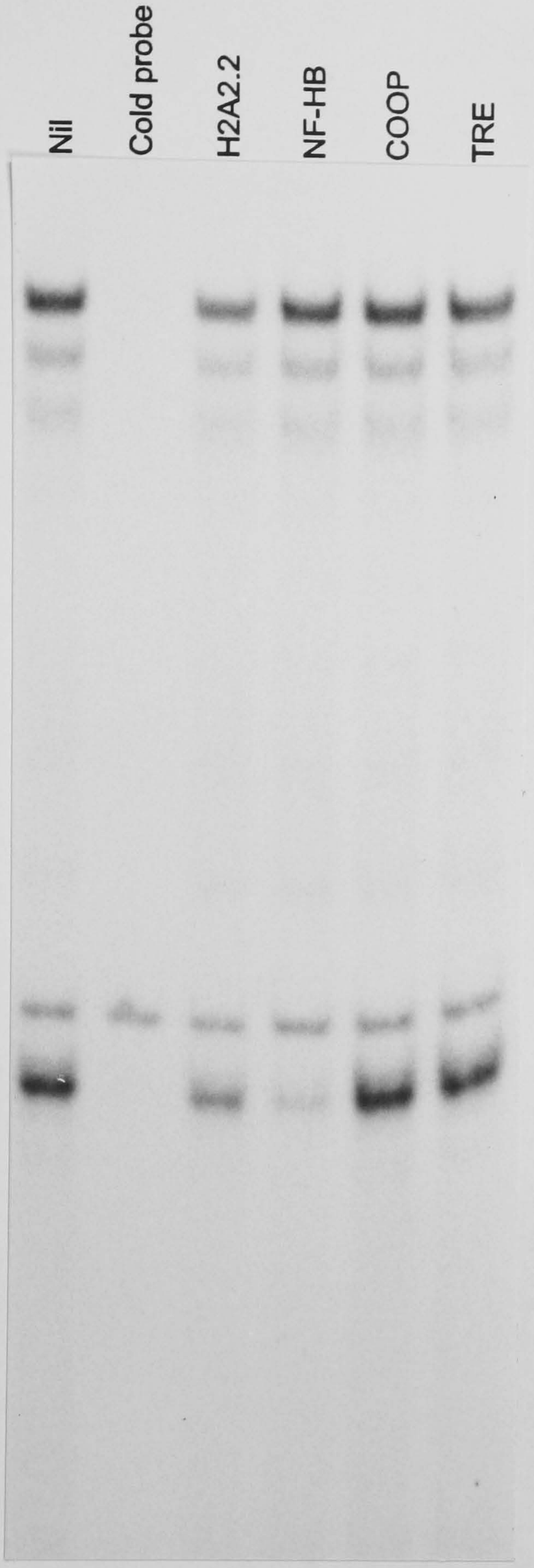


Figure 6.4. BSAP binds to the human ε–germline promoter. EMSA performed using a probe from 80-184 and nuclear extracts from unstimulated BJAB cells. The competing double-stranded oligonucleotides (100-fold molar excess) for the sea urchin H2A2.2 and murine NF-HB BSAP binding sites, and the unrelated transcription factors COUP, and TRE (Table 6.1), identify a putative BSAP complex (arrow).

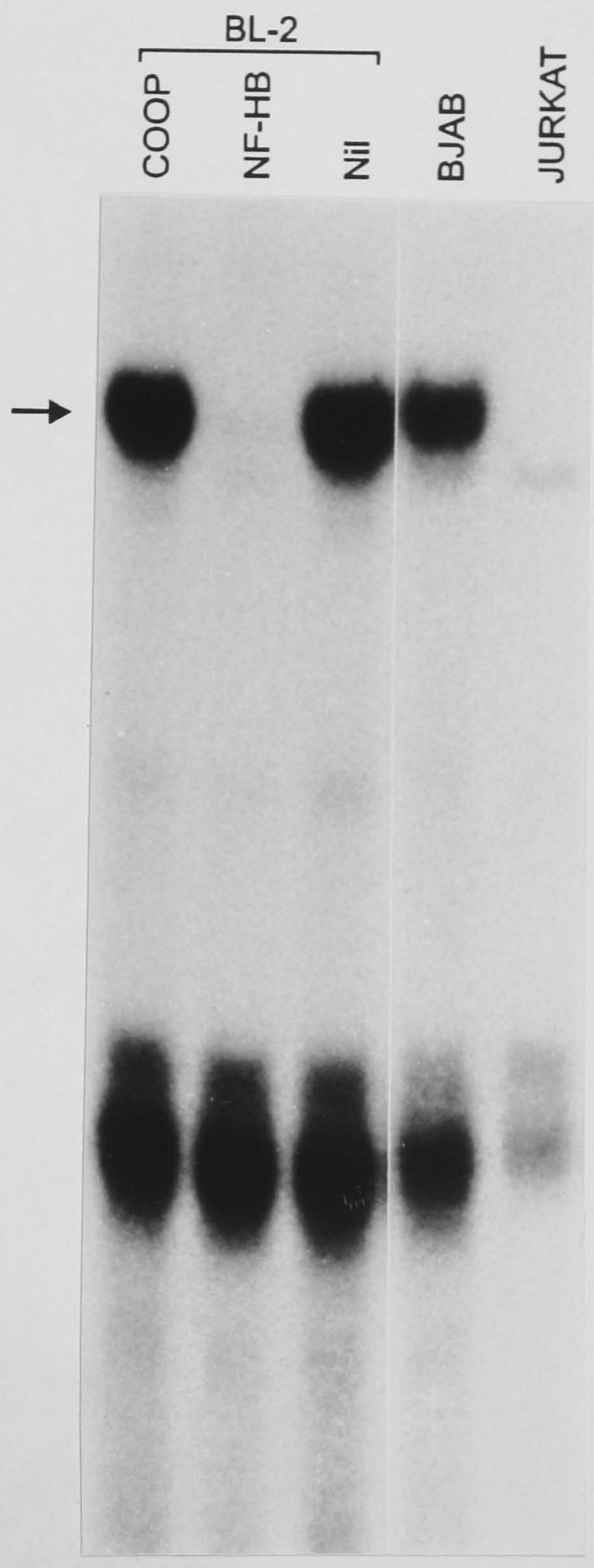


Figure 6.5. The putative BSAP complex is present in the B cell lines BJAB and BL-2 but not the T cell line Jurkat. EMSA with a probe from nucleotides 104-139 and extracts from BL-2, BJAB, and Jurkat. Competitors for the BSAP NF-HB site and the unrelated transcription factor COUP identify the position of the BSAP complex (arrow).

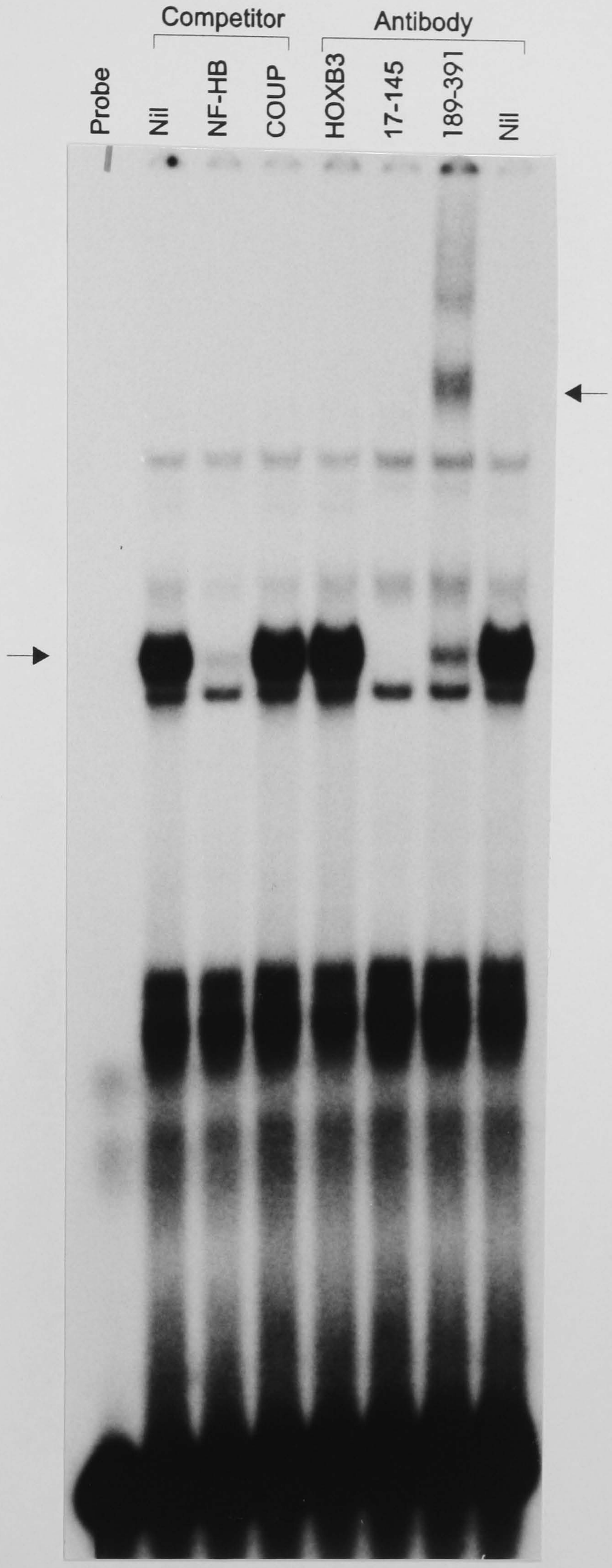


Figure 6.6. α–BSAP antibodies confirm the identity of the putative BSAP complex. EMSA using nuclear extracts from unstimulated BJAB cells and a probe (nucleotides 104-139) that spans the BSAP binding site in the ε-germline promoter. Rabbit antibodies specific for the paired (DNA binding) domain of BSAP (aa17-145) disrupt binding of BSAP (left arrow) to the probe. Antibodies specific the C-terminal region of the protein (aa189-391) supershift the BSAP band (right arrow). Negative control, antiserum to the unrelated transcription factor human HOXB3.

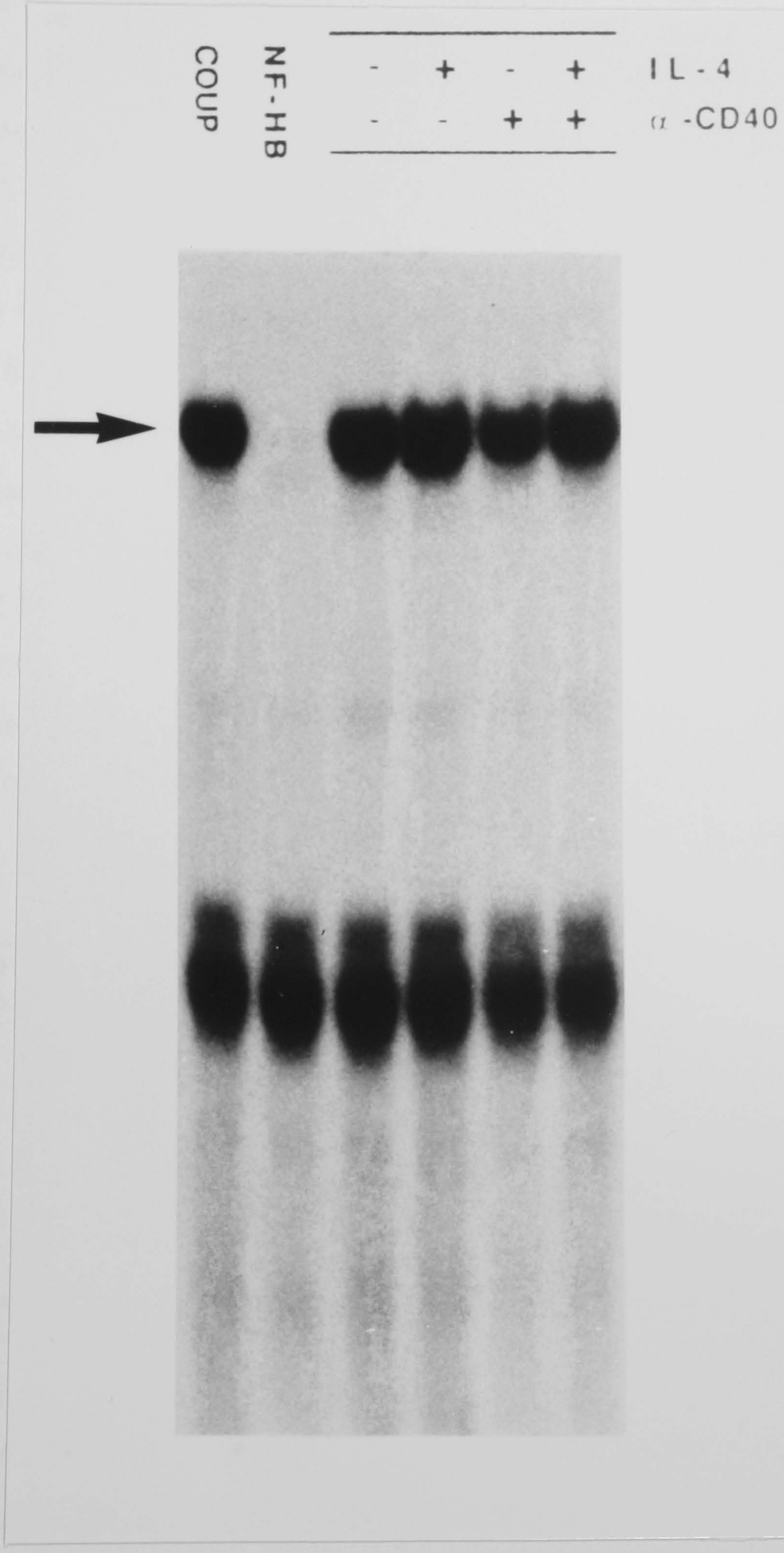


Figure 6.7. BSAP activity is not regulated by IL-4 or α-CD40 stimuli. EMSA analysis of BL-2 cells induced with IL-4, α-CD40, or both, using a probe from nucleotides104-139. Arrow, location of the BSAP complex.

6.2.3 An invariant C in the BSAP consensus sequence is essential for binding.

To determine if BSAP is essential for ε-germline transcription, we first searched for a mutation that could interfere with its binding to the Iε promoter. Of all known BSAP binding sites, only the C at position 8 of the core motif is invariant (Fig. 6.8). A point mutation (C→A) at the invariant C residue in a sea urchin H2A-2.2 BSAP site has been demonstrated to be sufficient to strongly impair the binding of murine BSAP in EMSA (Singh and Birshtein 1993; Liao *et al.* 1994). We tested whether an oligonucleotide containing the human Iε BSAP site with the C→A mutation could compete binding of human BSAP to an unmutated probe. Figure 6.9 illustrates that introduction of the C→A mutation reduced competition by approximately 5-fold compared with the unmutated competitor, over a 10-100X excess range. A G residue one base 5' to the invariant C is conserved among many of the BSAP binding sites (Fig. 6.8). When the conserved G residue was mutated to a T in addition to the C→A mutation, no further reduction in competition was observed. Thus, the invariant C in the BSAP site is important for *in vitro* DNA/protein interactions.

A

Site		1	2	3	4	5	6	7	8	9..10	11	*Reference*
NF-HB (core B)	T	G	T	G	A	A	G	C	G	T	G	(Liao *et al.* 1992)
NF-HB (core I)		T	G	T	G	A	A	G	C	T	T	A (Liao *et al.* 1992)
Sμ 96	G	C	T	G	-	A	G	C	T	G	A	(Xu *et al.* 1992)
Ig J chain	G	T	C	T	T	T	C	C	A	G	T	(Rinkenberger *et al.* 1996)
αS-1		A	G	T	G	T	A	G	C	A	A	G (Waters *et al.* 1989)
H2A-2.2		T	G	T	G	A	C	G	C	A	G	C (Barberis *et al.* 1989)
Lamdba 5		A	G	T	C	A	G	G	C	A	G	A (Okabe *et al.* 1992)
Murine Iε		A	C	T	G	A	G	G	C	A	G	A (Rothman *et al.* 1991)
CD19 site 1		A	G	A	C	A	C	C	C	A	T	G (Kozmik *et al.* 1992)
VpreB1		T	C	T	G	G	C	C	C	A	T	G (Okabe *et al.* 1992)
Consensus		D	N	H	B	D	N	S	C	D	D	N

B

BSAP Core

Human ACCCCATTTTTAGCTGAAAGCACTGAGGCAGAGCTCCCCCTACCCAGGCTCCA 147

Mouse ACCCCACTTTTAGCTGAGGGCACTGAGGCAGAGCGCCCCCTAGGTACTACCAT -8

Figure 6.8. BSAP binding sites. (*A*) BSAP core motifs (updated from Neurath et al. 1994). Dark grey box, the C present in all BSAP sites, and the G present in 7 of 10 motifs, chosen for point mutation. (*B*) Comparison of the human and mouse (Rothman et al. 1990) sequences surrounding the BSAP core motif (open box). Light grey, the additional 27 base deletion used in reporter constructs.

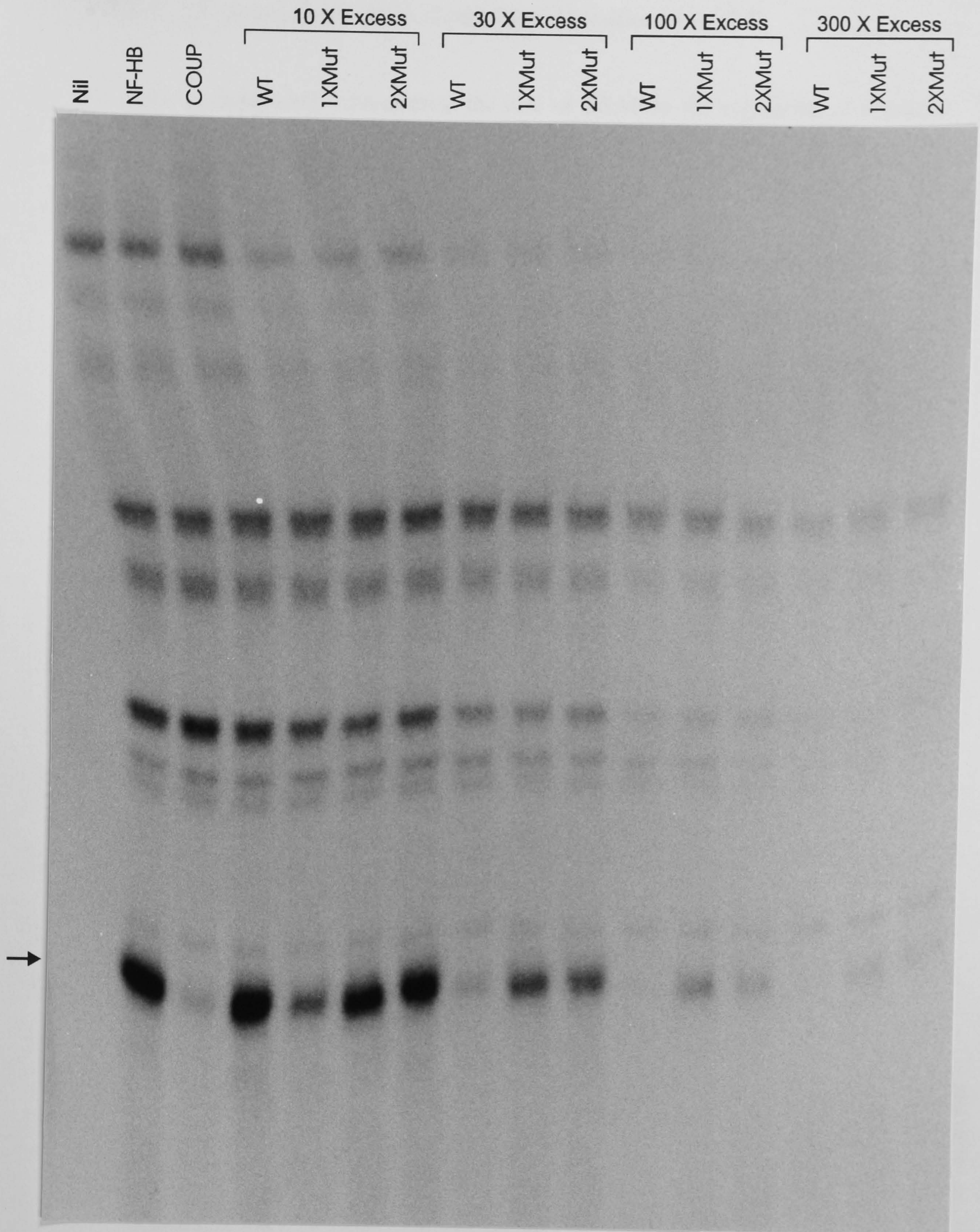


Figure 6.9. The invariant C in the BSAP binding site is essential for DNA/protein interactions. EMSA using BJAB nuclear extracts incubated with a wild-type BSAP probe (104-191, Fig. 6.2B). The BSAP complex (arrow) is competed much less efficiently by both the mutant, than the wild-type sites. All competitors used the same fragment (104-191) as the probe, mutated at the C or G (Fig. 6.8). The mut 1 competitor contains a C→A mutation at the invariant C in the BSAP binding site. Mut 2 contains both a C→A and a G→T mutation. Competitors were added at 10-, 30-, and 100-fold molar excess.

6.2.4 BSAP up-regulates the IL-4 and CD40 induction of ε-GLT.

In order to functionally characterise the role of BSAP in the regulation of human ε-germline transcription, reporter assays were performed using constructs that contain the human ε–germline promoter, with or without mutations in the BSAP binding site (Fig. 6.10). As a source of the promoter fragment, genomic DNA was amplified by polymerase chain reaction (PCR) using the ε-GLTREP primer pair (see Materials and Methods 2.18; Table 6.1). The human GL-ε promoter construct contained a 423 bp Rsa I-Hae II fragment of the amplified promoter region (bases -118 to 259, Fig. 6.2B) inserted into the promoterless pGL3 luc vector, and was confirmed by sequencing. Because a polymorphism exists in the GL-ε promoter at position 224 (Figs. 5.6, 6.2B), the IL-4 inducibility of the promoter constructs corresponding to both alleles was first compared in reporter assays. The two reporter constructs were transiently transfected at three DNA concentrations (30, 10, and 2.5 μg) into the cell line BJAB. After transfection, the cells were divided into aliquots, and incubated in the presence or absence of IL-4. Luciferase activity was assessed 48 hours later. A β–gal control plasmid was cotransfected with the reporter construct to normalise for transfection efficiency. In duplicate experiments, the mean level of expression in the presence of IL-4 varied from 2.0 to 3.4 fold over medium alone, but no relationship was seen between the polymorphism and differences in reporter activity (Fig. 6.11). The G allele at position 224 was used for the subsequent reporter studies. Preliminary experiments indicated that

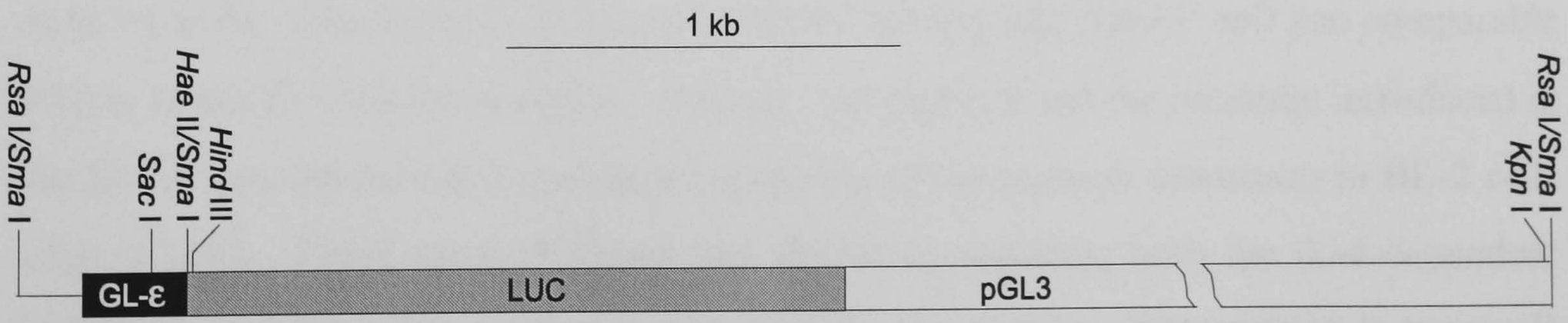


Figure 6.10. GL-ε Reporter constructs. The 423 base wild type germline promoter/exon *Rsa* I-*Hae* II fragment (bases -118 to 259) inserted into the *Sma* I site of pGL3. The BSAP mutations mut 1, mut 2, and the 27 base deletion were introduced into the parent pGL3-mutations mut 1, mut 2, and the 27 base deletion were introduced into the parent pGL3-GLε luciferase reporter vector.

expression of the luc constructs was inducible at higher and more consistent levels in BL-2 than in BJAB cells (Figs. 6.12, 6.13a). BL-2 cells were therefore used for this set of studies. The BSAP mut 1 and BSAP del constructs contained, respectively, the C→A mutation at nucleotide 123 (Fig. 6.8), and an additional 27 bp deletion encompassing the BSAP binding site (nucleotide 110-136). BSAP mut 1 was obtained by PCR amplification of the GL-ε reporter construct (G allele) with the REP1XMUT primer pair (see Materials and Methods 2.16; Table 6.1), after which a 312 bp Kpn I-Sac I fragment of the amplification product was replaced for the corresponding Kpn I-Sac I fragment of the GL-ε reporter construct (Fig. 6.10). The BSAP del construct was obtained by amplification of the GL-ε reporter construct insert in two half fragments with the BSAP Del 1 and BSAP Del 2 primer pairs (see Materials and Methods 2.16; Table 6.1). The two amplification products were then joined in a further PCR reaction with primers GL4 and Rvprimer3, and inserted into the pGL3 reporter vector as a 470 bp Kpn I-Hind III fragment (Fig. 6.10). BL-2 cells were transfected with the mut 1, BSAP del, and wild type promoter reporter constructs and with the reporter vector only (pGL3) as a negative control. After transfection, the cells were divided into aliquots, and incubated in the presence or absence of IL-4 and α–CD40 mAb. Figure 6.13a demonstrates that IL-4 stimulation of BL-2 cells transfected with the GL construct resulted in a 4.9-fold induction of luciferase activity. α–CD40 mAb strongly (14.9-fold) up-regulated transcription driven by the ε-germline promoter when added in combination with IL-4, but only slightly (1.6X) up-regulated transcription if added alone. The introduction of the C→A mutation in the BSAP binding site (BSAP mut 1) reduced the IL-4 inducibility of the promoter by >50%, and the response to the combination of IL-4 and the α–CD40 mAb by 65%. The deletion of the entire BSAP binding site (BSAP del) had comparable effects to the C→A point mutation. Notably, the deletion and the mutation introduced in the BSAP site did not affect the basal expression of the reporter constructs in BL-2 cells (Fig. 6.13B). These results indicate that BSAP up-regulates both the IL-4-dependent induction, and the CD40-mediated enhancement of ε-germline transcription in human B cells.

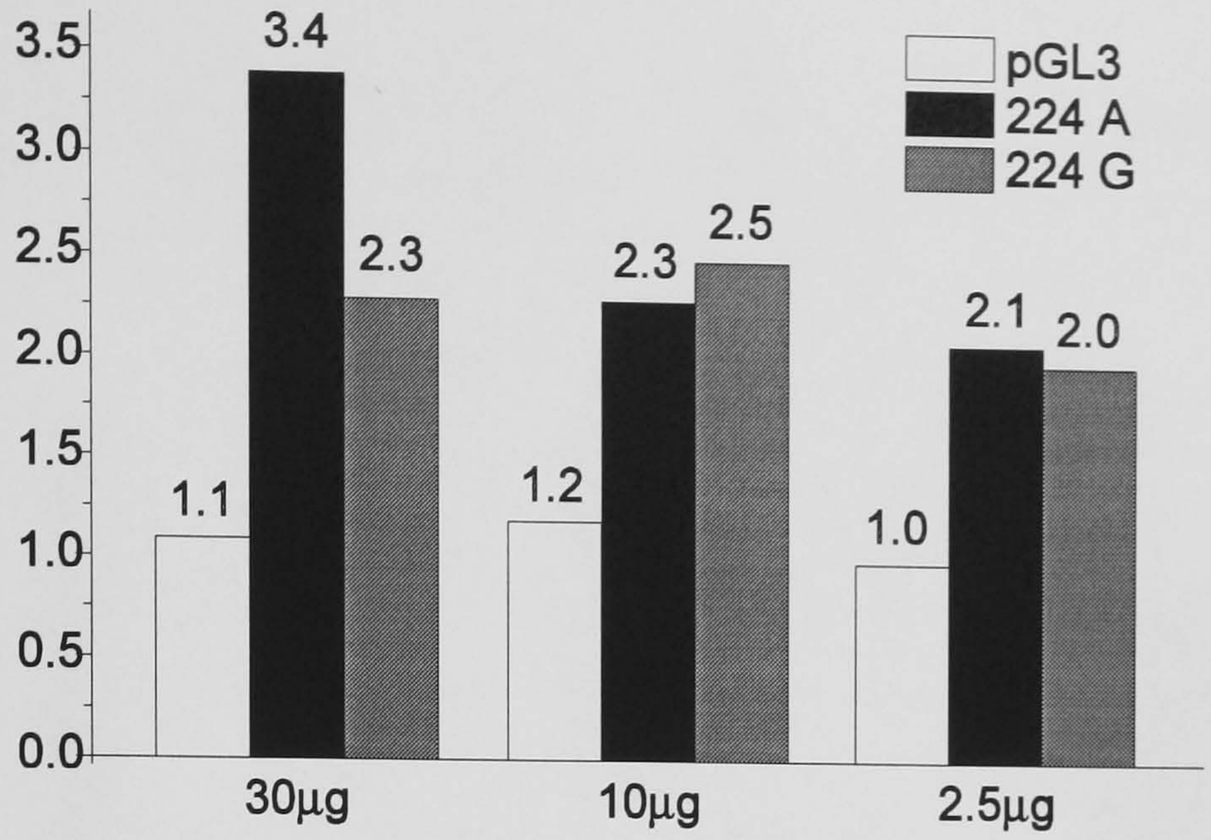


Figure 6.11. Affect of A/G polymorphism on reporter activity. Comparison of Reporter activity from both A and G alleles at position 224 (224 A, 224 G) in the GL-ε promoter construct (Fig. 6.10). BJAB cells were transiently transfected with three different amounts of each construct; 30, 10, and 2.5 µg. Vector only (pGL3) was used as a negative control. Results represent the mean of experiments performed in duplicate.

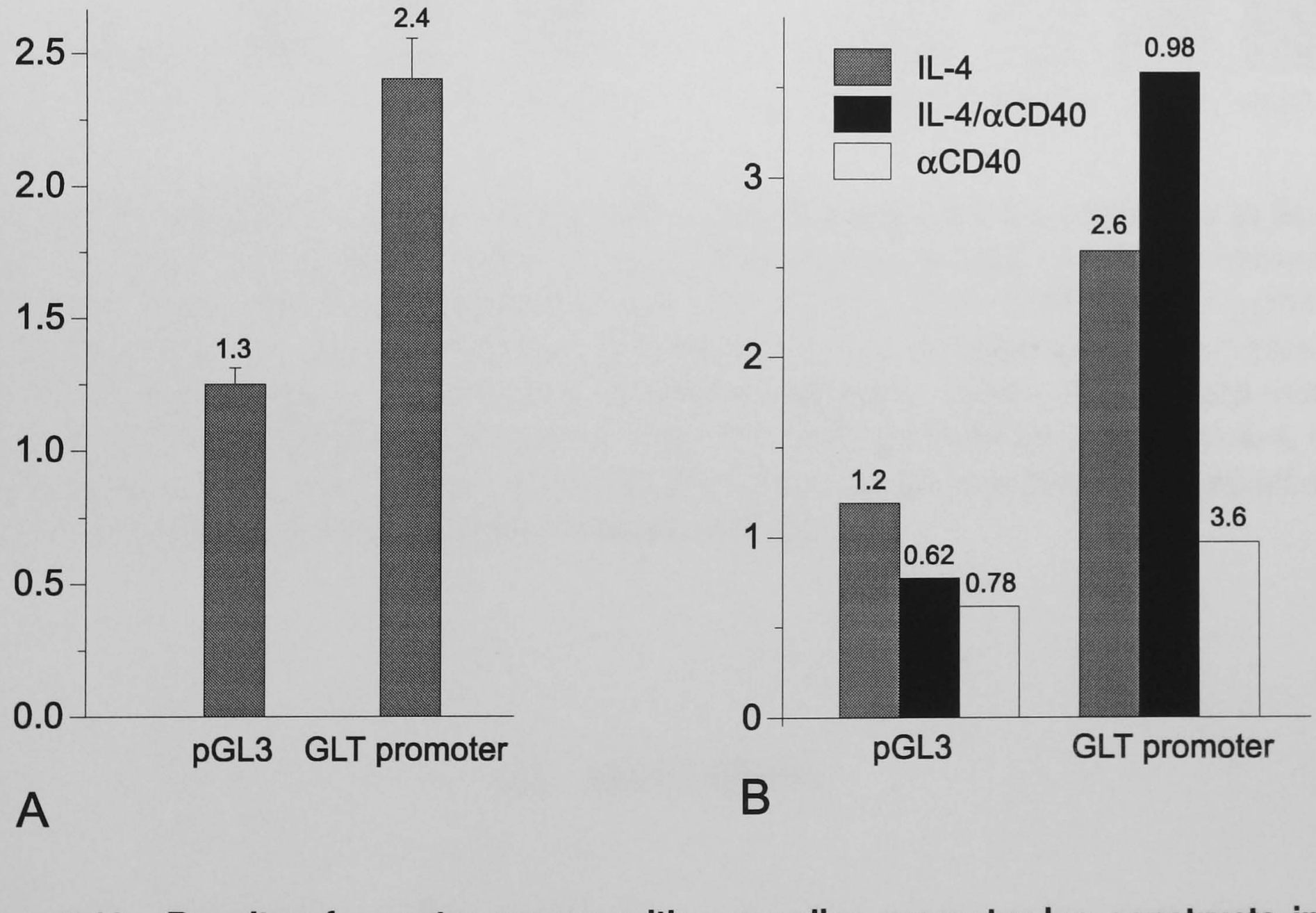


Figure 6.12. Results of reporter assays with ε-germline promoter luc constructs in BJAB cells. BJAB cells were transiently transfected using Diethylaminoethyl cellulose (DEAE)-dextran, and split into aliquots that were incubated in the presence or absence of IL-4 (100 U/mL) and α–CD40 mAb (5 µg/mL). Luciferase activity was assessed 48 hr later. (*A*) Fold induction with IL-4, (over medium alone) of the wild type GL-ε reporter (GLT promoter, Fig. 6.10) or the pGL3 vector only. Results are the mean and standard error of three experiments. (*B*) Fold induction in the presence of IL-4, αCD40, or IL-4/αCD40. Results are the mean of two experiments. A β–galactosidase control plasmid was cotransfected with the reporter construct, to normalise for transfection efficiency. A fold induction of 1.0 is equivalent to the expression level of the ε-germline promoter reporter construct in the presence of medium alone.

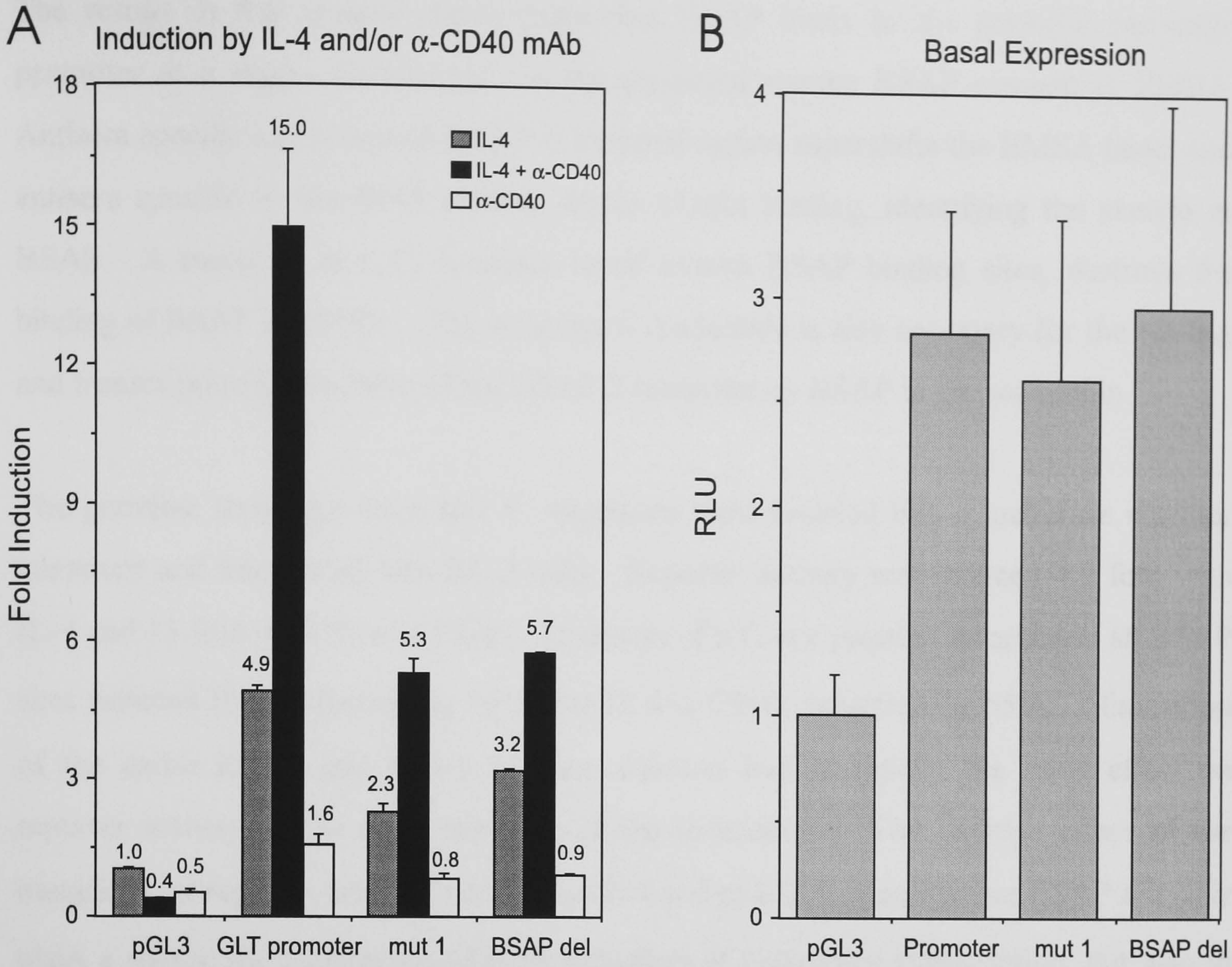


Figure 6.13. Results of reporter assays with ε–germline promoter luc constructs in BL-2 cells. BL-2 cells were transiently transfected using DEAE-dextran, and split into aliquots that were incubated in the presence or absence of IL-4 (100 U/mL) and α–CD40 mAb (5 μg/mL). Luciferase activity was assessed 48 hr later. A β–galactosidase control plasmid was cotransfected with the reporter construct, to normalise for transfection efficiency. Values obtained from mock transfections were subtracted as background. (*A*) fold induction of luciferase activity with IL-4, α-CD40 and IL-4/α-CD40 over medium alone. (*B*) Effect of the mutations on basal luciferase activity (no inducing agent), expressed in relative luciferase units (RLU).

6.3 DISCUSSION

Pax-5/BSAP is a B cell specific protein involved in the expression of a number of genes restricted to B cells, including CD19, λ5, $V_{pre}B1$, XBP 1, the tyrosine kinase *blk*, and the germline gene exons and switch regions of the IgH genes μ, γ1, γ2, ε, α. As the only known B cell specific protein that interacts with the GL-ε promoter it may represent a key regulatory factor that restricts the cell type expression of the germline transcript to B cells.

The results in this chapter demonstrate that BSAP binds to the germline transcript promoter at a region homologous to the proposed murine BSAP element in EMSA. Antisera specific to the human BSAP C terminal region supershifts the EMSA band, and antisera specific to the DNA binding region blocks binding, identifying the protein as BSAP. A mutation at a C, invariant in all known BSAP binding sites, destroys the binding of BSAP in EMSA. The invariant C nucleotide is also necessary for the binding and transcriptional activation of the H2A2.2 promoter by BSAP in the sea urchin.

The germline transcript exon and 5' sequences were inserted into a luciferase reporter construct and introduced into BL-2 cells. Reporter activity was induced 4.9 fold with IL-4 and 15 fold with IL-4/α-CD40. Mutation of a C at a position invariant in all BSAP sites reduced IL-4 induction by 50% and IL-4/α-CD40 induction by 65%. Elimination of the entire BSAP site with a 27 base deletion had essentially the same effect on reporter activity as the point mutation at the invariant C. The additive effect of the mutations on reporter activity induced by IL-4 and α-CD40 suggests that BSAP not only plays a role in the mechanism of IL-4 induction of ε-germline transcription, but also in the α-CD40 mediated up-regulation of transcription. Mutations in the BSAP binding site did not appreciably affect basal reporter activity suggesting that the role of BSAP in germline transcription is restricted to the up-regulation of transcription. As BSAP expression was unaffected by induction of BJAB cells with IL-4 and α-CD40, it is unlikely that the results seen in the reporter assays are influenced by the effect of the inducing agents on BSAP levels. The 50% and 65% reduction in the levels of IL-4 and IL-4/αCD40 induced reporter activity, respectively, indicates that although BSAP is involved in the regulation of ε-GLT, other factors function to initiate transcription in the reporter construct, though at a lower level of efficiency. A 65% reduction in germline transcription could markedly affect the level of switch recombination if a high level of ε-GLT may be required to maintain a chromatin environment appropriate for recombination. *In vivo,* in the presence of distal regulatory elements such as the immunoglobulin 3'-LCR, the effect of the BSAP mutation may be greater, since the reporter constructs could lack factors involved in communication between the GL-ε promoter and the LCR. Also, the lack of an appropriate chromatin environment in the reporter construct could affect some of the interactions required for the complete role of BSAP in ε-GLT.

IL-4 strongly induced ε-germline transcription in BJAB cells, whereas IL-4 alone normally induced little or no transcription in BL-2 cells. α-CD40 was required in combination with IL-4 to induce levels of germline transcription in BL-2 comparable to BJAB with IL-4 alone. In contrast, IL-4 alone mediated a strong induction of reporter activity in BL-2 cells (compare Figs. 6.1 to 6.13). One possible explanation for the high level of IL-4 induced reporter activity in BL-2 relative to endogenous germline transcription, is that the construct lacks negative elements which repress transcription from the endogenous gene in the absence of CD40 engagement. Derepression of the endogenous locus may require CD40 engagement, possibly mediated through the immunoglobulin 3' enhancer. The immunoglobulin 3' enhancer is known to be required for ε-germline transcription and switching in the mouse (Cogne *et al.* 1994). In BL-2 cells the 3' enhancer may be in a repressed state, exerting a repressive influence on the endogenous GL-promoter. As CD40 engagement is known to up-regulate the 3' enhancer (Grant *et al.* 1996), it could up-regulate expression of the endogenous germline transcript in BL-2 cells through activation of the enhancer. Conversely, BJAB cells could contain the 3' enhancer in a constitutively activated state enabling high level expression of the germline transcript after induction with IL-4 alone. In the absence of the repressive influence of the 3' enhancer on the reporter construct in BL-2 cells, IL-4 could induce high levels of promoter activity without CD40 engagement. The 3 fold enhancement of reporter activity in the presence of α-CD40 over IL-4 alone could involve a pathway independent of the 3' enhancer, such as through the activation of NF-κB (Francis *et al.* 1995).

Recent work reported by Albrecht *et al* (1996), concluded that BSAP was not involved in the IL-4 induced activation of human ε-germline gene transcription. Several differences in the experimental procedures of these authors from those used in this chapter could explain their different conclusions. In Albrecht *et al* the human Birkitt's lymphoma line DG75, and the murine B cell lymphoma M12.4.1 were used. The differentiation state of the cell lines used could have been before or after that needed to respond appropriately to transactivation by BSAP. Also, the mouse cell line used may not respond to BSAP or other factors necessary for transactivation of BSAP in the human expression construct, due to differences between the compliment of human and mouse transcription factors or their binding site affinities. The reporter constructs

contained promoter fragments that were 48 nucleotides shorter at the 3' end and 141 nucleotides at the 5' end. The regions absent in the constructs used by Albrecht *et al* (1996) could contain regulatory elements for factors needed to interact with BSAP. Also, a clone of the germline exon was used (Albrecht *et al.* 1994) that contains numerous differences in sequence from the genomic sequence determined in chapter 5 (Fig. 5.6), which could disrupt the binding of factors needed to interact with BSAP. Additionally, the reporter vector used by Albrecht *et al*, pGL2, is known contain sequences that target the luciferase protein to peroxisomes, that could affect expression levels through toxic effects (Groskreutz *et al.* 1995). The later pGL3 series of reporter vectors used in this chapter overcame this problem.

BSAP clearly plays a role in the transcriptional up-regulation of ε-germline transcription through up-regulate binding site in the promoter. BSAP may also regulate germline transcription through interaction with the 3' enhancer and the control of proliferation. BSAP may mediate some of these functions through interactions with other proteins involved in GL-ε transcription and switching, *e.g.* interaction with Ets family members in the mb-1 promoter and octamer binding proteins in the 3' enhancer. Given the interaction of BSAP with Ets family members in the mb-1 promoter and its potential interaction with the Ets family member NF-αP in the immunoglobulin 3' enhancer, the possibility exists that BSAP may mediate communication between the germline promoter and the enhancer through BSAP-Ets interactions. Several Ets family members are known to bind the 3' enhancer (Grant *et al.* 1995; Linderson *et al.* 1997), any one of which could mediate BSAP-Ets interactions with the ε–germline promoter. Corcoran, *et al* (1998) have also suggested a role for BSAP in the mediation of chromatin structure and germline gene transcription in the immunoglobulin heavy chain variable region gene segments. Mice lacking the alpha-chain of the interleukin-7 receptor are progressively impaired with distance, in V segment recombination and germline gene transcription. Coincident with the impairment in recombination is a decrease in BSAP expression, suggesting a role of BSAP in V region chromatin structure through its activity on the immunoglobulin LCR.

CHAPTER 7: Mechanism of the Enhancement of IgE Synthesis by Glucocorticoids.

7.1 INTRODUCTION

The association of high serum levels of IgE with allergic disease is well accepted (Leung 1993). Similarly, levels of allergen specific IgE increase in allergic rhinitics during exposure to allergen. Corticosteroids are the primary treatment for the symptoms of rhinitis, but the exact mechanism(s) of their action are largely unknown (for review see Schleimer 1996; Schwiebert *et al.* 1996).

Glucocorticoid treatment has been shown to diminish the seasonal rise in levels of allergen specific IgE (Crimi *et al.* 1990; Naclerio *et al.* 1993). In the local environment, B cells in the nasal mucosa of rhinitics increase their expression of IgE mRNA after allergen challenge, and this increase is inhibited by topical glucocorticoid (Durham *et al.* 1997). Total serum IgE levels, conversely are unchanged or increase moderately after glucocorticoid treatment (Gunnar *et al.* 1970; Henderson *et al.* 1973; Posey *et al.* 1978; Settipane *et al.* 1978; Klebl *et al.* 1994; Zieg *et al.* 1994). Furthermore studies have demonstrated that the effect of glucocorticoids on total IgE is more pronounced *in vitro*. Glucocorticoids significantly increase IL-4 induced total IgE levels in cultures of peripheral blood mononuclear cells (Wu *et al.* 1991; Nusslein *et al.* 1994).

Little is known about the mechanism of the up regulation of IgE synthesis by glucocorticoids. IgE germline transcription occurs as soon as 12 hours after IL-4 addition, a functional epsilon transcript is produced after heavy chain gene recombination at 4-6 days, and secreted IgE appears at ≈8 days. Enhancement still occurs when glucocorticoid is added as late as day 6 after initiation of the culture (Wu *et al.* 1991), suggesting it still acts at the IL-4 independent stage of the IgE response. Also, spontaneous IgE secretion is increased by glucocorticoids in cultures of peripheral blood mononuclear cells in the absence of IL-4 (Kimata *et al.* 1995; Hiratsuka *et al.* 1996). Pre-stimulation of PBMC with glucocorticoid prior to addition of IL-4 was also sufficient to enhance IgE synthesis (Fischer and Konig 1990). The *in vitro* potentiation of IL-4 induced IgE secretion by glucocorticoids is monocyte dependent (Wu *et al.* 1991). The enhancement activity of glucocorticoids therefore is not dependent on IL-4 and acts in a time independent manner in the first 6 days of culture. In purified B cell cultures

hydrocortisone act as a co-stimulatory signal to induce switching to IgE in the presence of IL-4 (Jabara *et al.* 1991; Jabara *et al.* 1993). In the presence of IL-4, hydrocortisone up-regulated the level of ε-germline transcription 2 fold in purified B cell cultures and induced production of the VDJ-containing Cε transcript. The potentiation of IgE synthesis in PBMC cultures may occur by a different mechanism than with purified B cell cultures. When glucocorticoid is added to purified B cells in the presence of IL-4 it appears to replace the cell contact-mediated signal for class switching, and unlike its effect on the potentiation of IgE synthesis in PBMC, it is not monocyte dependent but requires glucocorticoid at the onset of culture (Jabara *et al.* 1991; Jabara *et al.* 1993).

FcεRII plays a central role in IgE regulation. Similar to IgE, serum levels of FcεRII also increase in relation to atopy. Glucocorticoids down-regulate the unstimulated and IL-4 induced expression of membrane and secreted FcεRII from PBMC, B cells (Fischer and Konig 1990; Wu *et al.* 1991; Paterson *et al.* 1994), monocytes, and eosinophils (Kawabe *et al.* 1991), *in vitro*. This suppression of FcεRII expression is blocked by EBV and CD40 engagement (Katira *et al.* 1993; Paterson *et al.* 1994).

Glucocorticoids cause an increase in the apoptosis of B cells (Holder *et al.* 1992; Merino *et al.* 1994), T cells (Perandones *et al.* 1993), and eosinophils (Matsukura *et al.* 1997), and rescues neutrophils from apoptosis (Cox 1995; Kato *et al.* 1995). Recombinant soluble FcεRII rescues germinal B cells from apoptosis (Bonnefoy *et al.* 1993), but membrane CD23 cross-linking induces apoptosis in resting B cells (Campbell *et al.* 1997). High levels of FcεRII suppress IgE synthesis, potentially through apoptosis or effects on B cell differentiation (Cho *et al.* 1997).

Glucocorticoids inhibit many cytokines in peripheral blood mononuclear cells, including IL-2, IL-3, IL-4, IL-5, IL-10, IL-12 and interferon-gamma (IFN-γ) (Brinkmann and Kristofic 1995; Barnes 1996; Brattsand and Linden 1996; Elenkov *et al.* 1996). Both IFN-γ and IL-12 inhibit IL-4 induced IgE synthesis in PBMC (Pene *et al.* 1988; Kiniwa *et al.* 1992), most likely through their ability to induce uncommitted T helper cells to a TH1 phenotype.

The activity of glucocorticoids in allergy are reminiscent of those of the steroid hormone vitamin D3. Vitamin D3 inhibits IgE-mediated biphasic cutaneous reactions (Katayama *et al.* 1996). Like glucocorticoids vitamin D3 suppresses FcεRII expression (Fargeas *et al.* 1990; Boltz-Nitulescu *et al.* 1995). Vitamin D3 also inhibits NF-κB activity (Yu *et al.* 1995). Vitamin D3 inhibits the expression of all three subtypes of major histocompatability complex class-II antigens on monocytes (HLA-DR, -DP and -DQ) as well as the accessory activity of monocytes in promoting T cell activation, and IL-4 addition reverts these effects (Rigby and Waugh 1992; Xu *et al.* 1993). The similarities between the activities of glucocorticoids and vitamin D3 raise the possibility that both steroid hormones share some of the same mechanisms of action.

The apparent contradiction between the up-regulation of IgE levels by glucocorticoids with the beneficial effects of these drugs on allergic symptoms warrants further investigation. Knowledge of the mechanism of the enhancement of IgE synthesis by glucocorticoids could help to reduce this potential antagonism to the efficacy of this drug in the treatment of allergic symptoms. Knowledge of the mechanism of glucocorticoid potentiation of IgE levels could also help to further elucidate the IgE regulatory network. Because the glucocorticoid hydrocortisone provides a co-stimulatory signal with IL-4 to induce germline and productive epsilon transcription and switching to IgE in purified B cells, the possibility that in PBMC, glucocorticoids also enhance these transcription events and the level of heavy chain switching to IgE is investigated in this chapter.

7.2 RESULTS

7.2.1 Fluticasone potentiates IgE secretion *in vitro*.

Several possibilities were explored to elucidate the mechanism of the glucocorticoid potentiation. The glucocorticoid fluticasone 17-propionate was used for these studies. Initially, if the effect of fluticasone on the germline gene and productive transcript levels for IgE was investigated. If fluticasone acts at a point before or during heavy chain switching then GL-ε transcript levels are expected to be greater in the presence of fluticasone. However, if fluticasone acts to up-regulate productive (VDJ-Cε)

transcription, or at a point in the mechanism of heavy chain switching after the induction of germline transcription, VDJ-Cε transcript levels should be greater in the presence of fluticasone. If fluticasone does not up-regulate productive (VDJ-Cε) transcription, then it could act by up-regulating the secretion of IgE or by affecting the ratio of secreted vs membrane bound IgE levels.

Atopic patients were screened for the fluticasone experiments by two criterion: 1) A 2 year history of hay fever. 2) A positive allergic skin prick test (greater than 5 mm weal) to Timothy grass pollen extract (*Phleum pratentase*, Soluprick, ALK, Denmark). PBMC were isolated from the venous blood of three atopic volunteers, and incubated with and without IL-4, and various concentrations of fluticasone 17-propionate. Parallel cultures were harvested at 4.5 days for RNA analysis, when both the germline and productive transcripts are present, and 14 days for measurement of secreted immunoglobulin, when secreted IgE levels are at a maximum.

IgE was induced in the presence of IL-4 alone in PBMC from two of the three atopic donors (TW and SD, Figs. 7.1A and 7.1B), and in the third (NP), IgE was below the level of detection (Fig. 7.1C). Fluticasone enhanced the level of IgE secreted from donors SD and TW at concentrations from 10^{-6}M to 10^{-12}M, and 10^{-6} to 10^{-10}M respectively, with a maximum enhancement of ≈5 fold over IL-4 alone in both donors. PBMC from NP, which produced no detectable IgE with IL-4 alone (Fig. 7.1C), secreted IgE with addition of 10^{-6}M to 10^{-9}M fluticasone at levels reaching a maximum similar to the donors SD and TW.

Secreted IgE was not detected in the presence of fluticasone alone except in the case of TW, which produced a moderate amount of IgE at 10^{-7} and 10^{-10}M fluticasone (Fig. 7.1A). Induction of IgE in the absence of IL-4 in this case was probably due to the production of high endogenous levels of IL-4 from a greater than normal level of preactivated mononuclear cells. Spontaneous production of IgE occurs occasionally in some cultures, and could be due to the immunological status of the donor at the time of the experiment. When the protein synthesis inhibitor emetine (5 μg/mL) was added at the onset of culture, no IgE was produced (Fig. 7.1), showing that the effect of fluticasone did not result from preformed IgE.

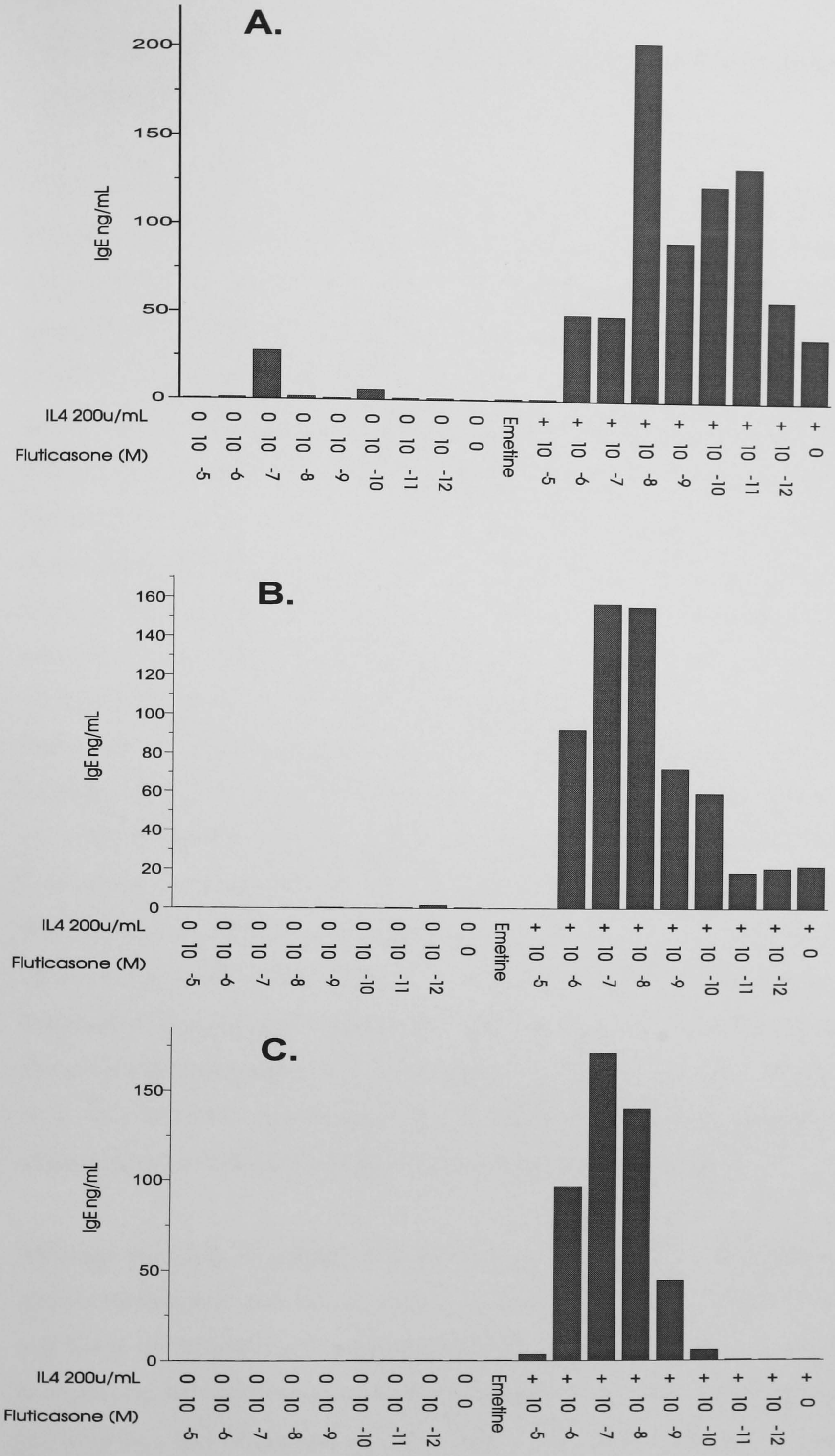


Figure 7.1. Fluticasone potentiates IL-4 induced IgE synthesis. ELISA analysis of secreted IgE levels from cultures of PBMC incubated 14 days with or without IL-4 and fluticasone. PBMC were isolated from (*A*) Steve Durham (SD), (*B*) Timothy Whitfield (TW), and (*C*) Nick Powel (NP).

7.2.2 GL-ε and VDJ-Cε transcript levels were not increased by fluticasone early in the culture period.

RNA was extracted from parallel cultures induced for 4.5 days, and levels of the ε-germline transcript, VDJ-Cε, and the secreted form of Cε were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). The transcript levels of each assay were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to adjust for loading error. Since GAPDH is expressed in all cell types present in the culture, we adjusted the GAPDH to account for the percentage of cells represented by B cell types, *e.g.* 40% B cells represents 40% of the total GAPDH value. An adjustment for the B cell percentage allows changes in the populations of the different cell types in the PBMC sample to be taken into account, due to the affects of the inducing agents on proliferation or cell death. An increase of ε germline transcript levels of 0.5 to 2.5 fold occurred in the presence of fluticasone and IL-4 (Fig 7.2), compared to IL-4 alone. When the PBMC were analysed by FACS at day 6 and 7 (Fig. 7.5), we discovered that the percentage of B cells present in the total population increased about 2 fold in the presence of fluticasone and IL-4, when compared to IL-4 alone. If the fold increase of ε-GLT measured by RT-PCR was corrected by the change in the B cell contribution (two-fold increase in the B cell percentage seen in Figure 7.5A and 7.5B) to the GAPDH level, germline-ε transcript levels per cell do not appreciably increase. At the same time, VDJ-Cε transcript levels increased 1.7 to 3 fold compared to IL-4 alone (Fig. 7.3). Again, if the levels of the VDJ-Cε transcript are corrected by the change in the B cell contribution, the increase is not reflected on a per B cell basis. Even if the correction for the change in B cell percentages were not made for the ε-GLT and the VDJ-Cε levels, the 0.5 to 3 fold increase in transcript levels would not reflect the greater than 450 fold enhancement by fluticasone of secreted IgE from the NP induction.

Although the levels of germline and productive transcripts did not change appreciably after glucocorticoid treatment, the increase in secreted IgE could be caused by a shift from the expression of the membrane to the secreted form of IgE. An increase in the ratio of secreted to membrane IgE levels could occur without changing the level of total IgE mRNA. To test the possibility that fluticasone caused a shift in the ratio of secreted to membrane epsilon transcripts, while not affecting total VDJ-Cε or GL-ε transcript levels, secreted Cε containing transcript levels were also measured. Secreted Cε containing transcript levels increased up to only 1.0 to 1.6 fold over IL-4 alone (Fig. 7.4), without adjusting for an increase in the B cell percentage, strongly suggesting that fluticasone did not shift the ratio of mRNA isoforms to secreted IgE.

Table 7.1 Oligonucleotides used in this study.

Pair Name	Primer Pair (upper strand, lower strand)	Size[a]	T°[b]	Reference/*Accession*
ε-GLT	CCACAGGCACCAAATGGACGAC	396	62°C	Fig. 5.7 nt 220-256
	CAGGACGACTGTAAGATCTTCACG			Fig. 5.7 nt 611-634
VDJ-Cε	GGTCAC(CT)GTCTCCTCAGCCTC	381	62°C	(Tomlinson et al. 1996)
	CAGGACGACTGTAAGATCTTCACG			Fig. 5.7 nt 611-634
Classic (ε-Secreted)	CCCCACTGCACAGCTGGATG	404	64°C	(Zhang et al. 1994)
	GCGGTCCACGACCAAGACCAG			*J00222* nt 2434-2454
M2" (ε-M2")	AGCCACCCCTCCTCGATGAC	346	64°C	(Zhang et al. 1994)
	GCGGTCCACGACCAAGACCAG			*J00222* nt 2434-2454
GAPDH	GGGAAGGTGAAGGTCGGAGTC	438	58°C	*M33197* nt 64-84
	CTGATGATCTTGAGGCTGTTG			*M33197* nt 483-503

[a]Size of PCR product. [b]Annealing temperature used for PCR amplification.

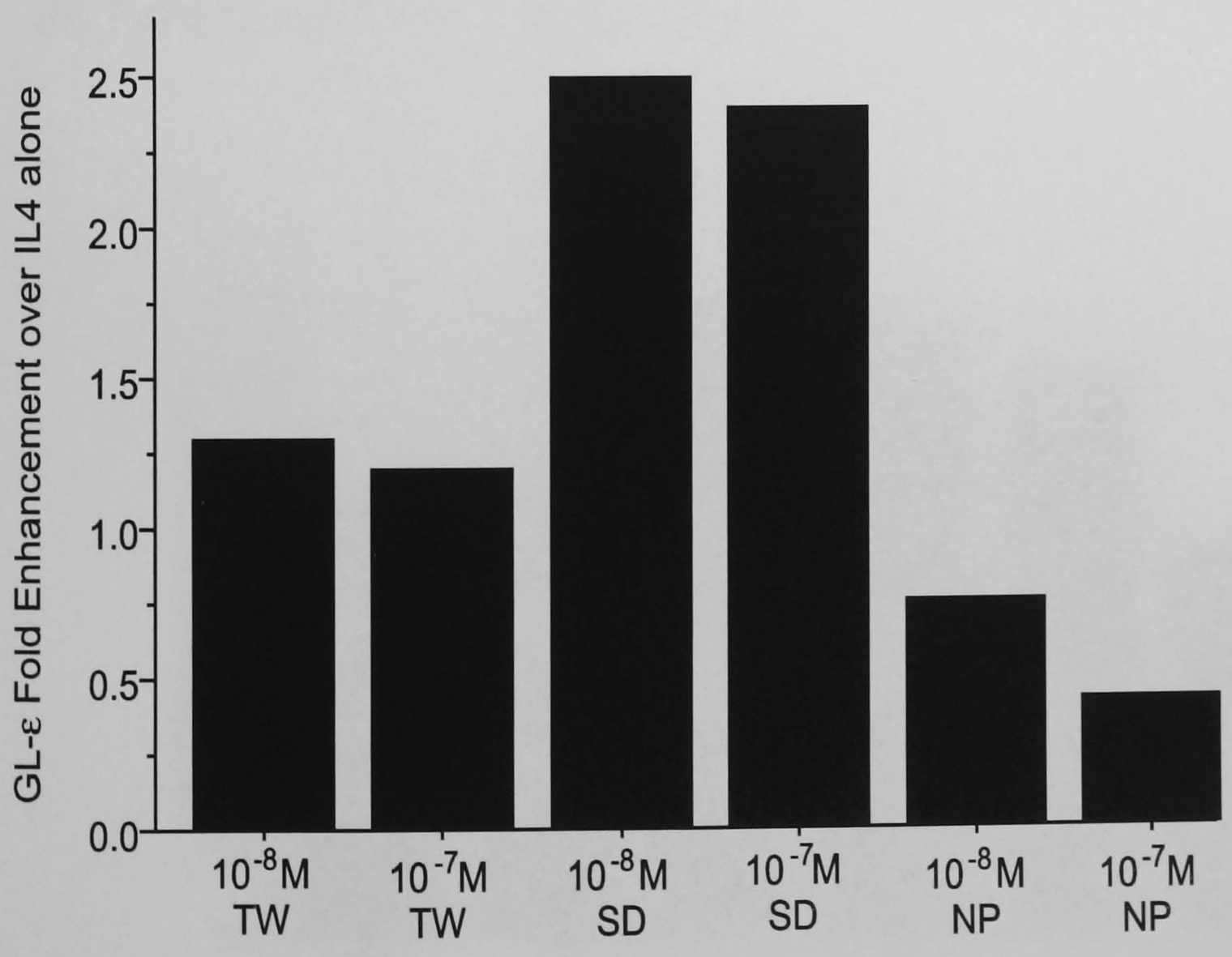


Figure 7.2. Effect of fluticasone on IL-4 induced ε-Germline gene transcript levels. RT-PCR analysis of PBMC incubated with IL-4 and 10^{-7} or 10^{-8}M fluticasone, using PCR primers specific for the ε-GLT (Table 7.1). Fold enhancement by fluticasone compared to IL-4 alone is shown. Results are quantified using phosphoimagery. NP, Nick Powell; SD, Steve Durham; TW, Timothy Whitfield.

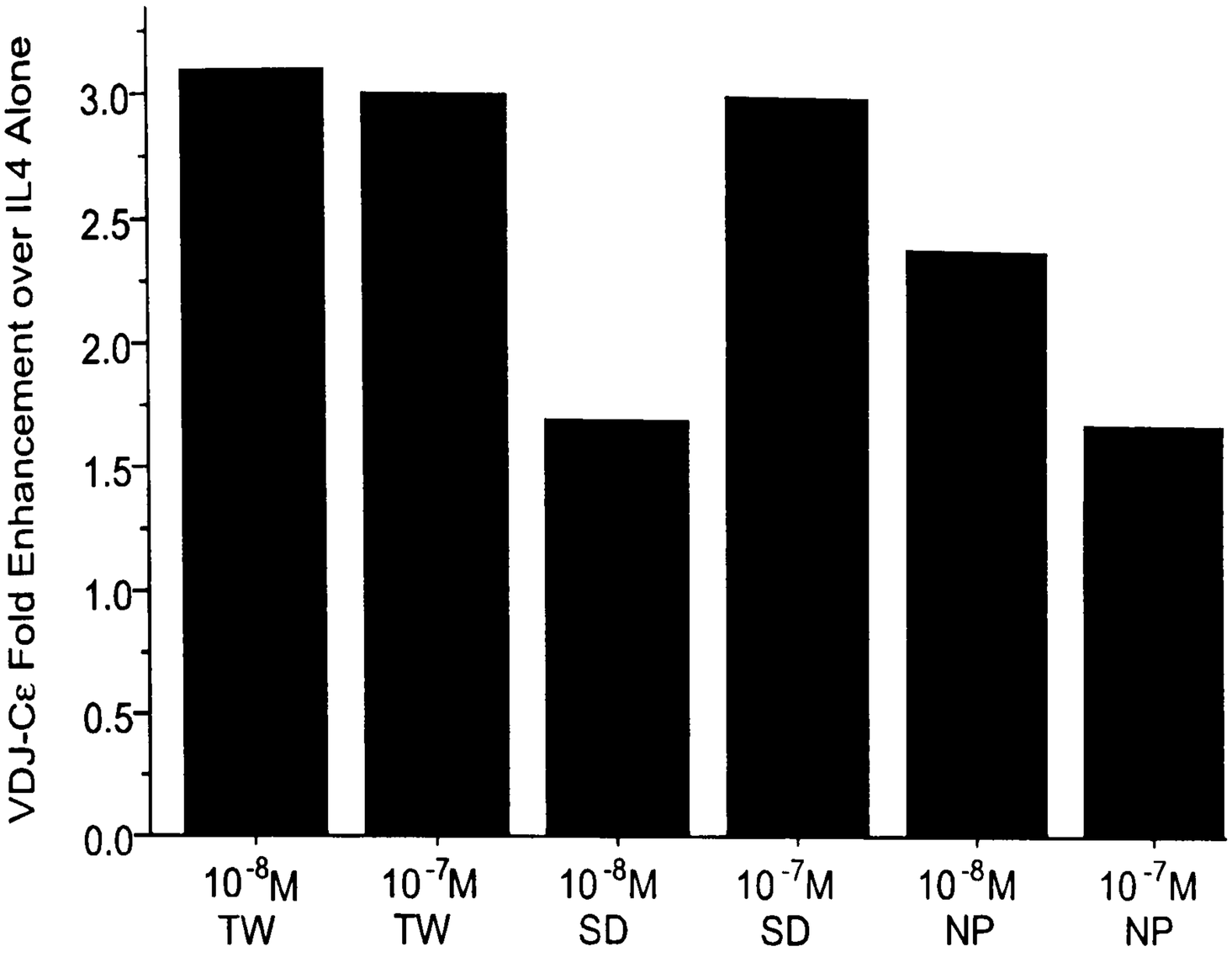


Figure 7.3. Effect of fluticasone on IL-4 induced VDJ-Cε transcript levels. RT-PCR analysis of PBMC incubated with IL-4 and 10^{-7} or 10^{-8}M fluticasone, using PCR primers specific for the VDJ-Cε transcript (Table 7.1). Fold enhancement by fluticasone compared to IL-4 alone is shown. Results are quantified using phosphoimagery. NP, Nick Powell; SD, Steve Durham; TW, Timothy Whitfield.

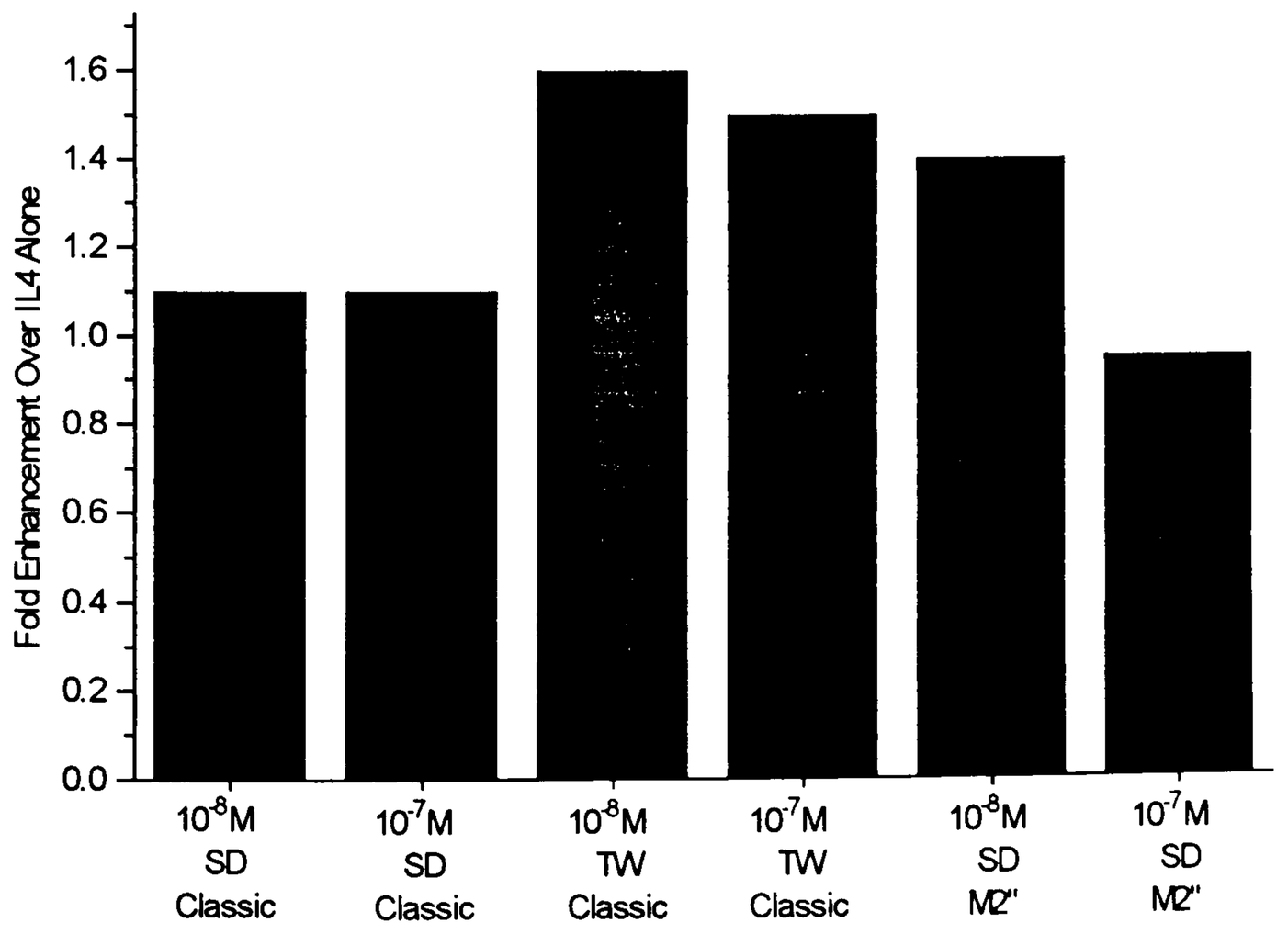


Figure 7.4. Effect of fluticasone on secreted/membrane epsilon transcript ratios. RT-PCR analysis of PBMC incubated with IL-4 and 10^{-7} or 10^{-8}M fluticasone, using oligonucleotides specific for the two main secreted epsilon mRNAs. Both the classic and M2" primer pairs detect mRNAs that encode secreted epsilon isoforms (Table 7.1). Fold enhancement by fluticasone compared to IL-4 alone is shown. Results are quantified using phosphoimagery. NP, Nick Powell; SD, Steve Durham; TW, Timothy Whitfield.

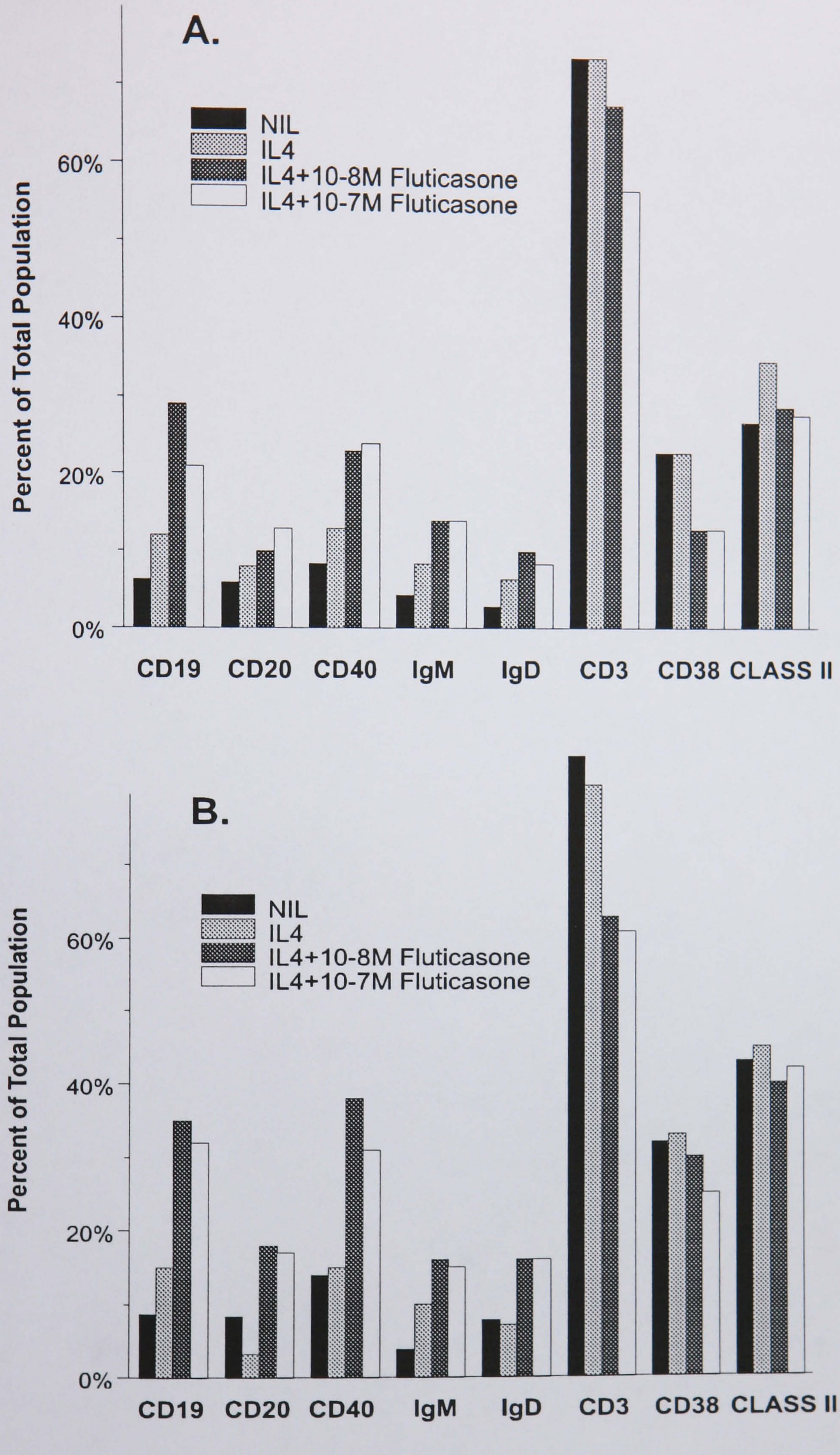


Figure 7.5. B cell percentage rises after fluticasone treatment. FACS analysis of cells harvested at (*A*) day 6, or (*B*) day 7, stained with a panel of B cell specific antibodies, against CD19, CD20, CD40, IgM, and IgD; the T cell specific antibody against CD3; MHC class II, or the antibodies against B cells + activated T cells, CD38. (*A*) Donor OMK, (*B*) Rachel Mallalien

7.2.3 The *in vitro* potentiation of IgE by fluticasone occurs after 10 days.

Since fluticasone is not required before day 6 of culture, the time at which fluticasone caused an increase in IgE levels over IL-4 alone was determined. PBMC from an allergic donor (SD) were incubated with IL-4 and fluticasone for 6, 8, 10, 12, and 15 days, and assayed for secreted IgE. A slight but measurable level of IgE was detected at 6 days of culture in the presence of IL-4 (Fig 7.6). At 6 days IgE levels in the presence of IL-4 and fluticasone were near background. At day 8 IgE production increased in the presence of IL-4, and IL-4 with the lowest concentration of fluticasone (10^{-8}M), but was lower at the highest level of fluticasone (10^{-7}M). Secreted IgE appeared to reach the same level between IL-4, and IL-4 with fluticasone at day 10. Interestingly, at day 12 and 15, IgE produced by IL-4 treated cultures appeared not to change from day 10, but in the presence of fluticasone IgE levels continued to rise until day 15.

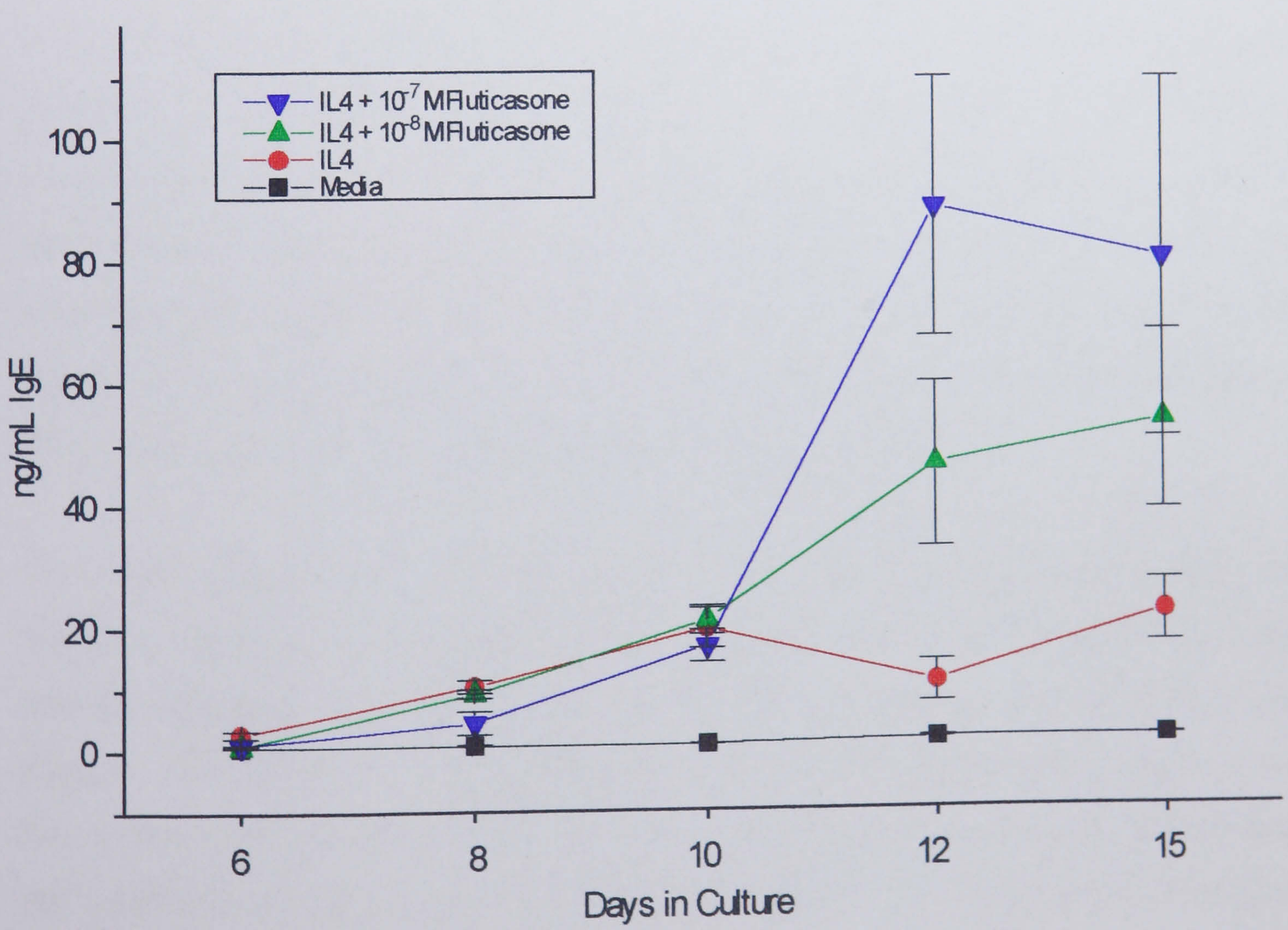


Figure 7.6. Fluticasone potentiation occurs late in culture. ELISA analysis of secreted IgE levels from cultures of PBMC incubated for 6-15 days with or without IL-4 and fluticasone. All cultures were performed in triplicate, and IgE levels are expressed with standard error. Donor, Steve Durham.

7.3 DISCUSSION

Fluticasone potentiated IL-4 induced IgE synthesis at least 5 fold in cultures of PBMC. Epsilon germline gene transcript levels were not significantly increased at day 4.5 of culture in the presence of fluticasone. Germline transcription is expected to be at a maximum at 4.5 days, and is a required event immediately prior to switching. The lack of an effect on germline transcription suggests the enhancement of IgE synthesis was not the result of an increase in heavy chain switching. VDJ-Cε germline transcript levels did not increase significantly at day 4.5 of culture supporting the conclusion that switching is not affected by fluticasone. Also, the ratio of membrane to secreted IgE mRNA did not change at day 4.5. This excluded the possibility that although total IgE remains constant, a reduction in the membrane form and increase in the secreted form of IgE mRNA caused the increase in secreted IgE. The possibility remains that VDJ-Cε transcription or the secreted to membrane ratio increases at a time later than 4.5 days, due to events caused by an effect on cell differentiation induced by fluticasone.

If the potentiation of IgE synthesis by glucocorticoid does not occur until after 10 days of culture, as was revealed in these experiments, a comparison of GL-ε and VDJ-Cε transcription at days 4.5, 10, and 15, in the presence and absence of glucocorticoids could reveal an effect on transcription at later time points. The ability of glucocorticoids to co-stimulate ε–germline transcription and switching to IgE in purified B cell cultures (Jabara *et al.* 1993) suggests that an effect on transcription should be seen also in PBMC, though potentially at later time points than were tested.

The effect of glucocorticoids could cause a prolonged period of competency for switching. If the period of switching was extended, the level of epsilon mRNAs may not increase until later in the culture period, after the 4.5 day time point assayed in this chapter. Also, glucocorticoids might induce switching through a differentiation event that occurs later than the events induced by IL-4 alone. Glucocorticoids could induce IgE synthesis through a mechanism independent of the IL-4/CD40/CD40-ligand mediated mechanism occurring in the presence of IL-4 alone. The initial levels of IgE produced starting at day 8 of culture could be induced by IL-4/CD40/CD40-ligand dependent mechanism both in the presence and absence of glucocorticoids. After Day 10

the effects of glucocorticoids may cause an additional cellular differentiation event that co-stimulates switching to IgE at a later time than in the presence of IL-4 alone. The later, glucocorticoid induced differentiation event, could occur through the same mechanism as for the induction of switching in purified B cells by hydrocortisone and IL-4. Additional work is needed to determine if glucocorticoids mediate a previously un-described co-stimulatory mechanism for switching to IgE.

To determine whether the effects of fluticasone occur though an up-regulation of switching, an increase in the number of cells expressing IgE can be determined by an enzyme-linked immunospot assay (Czerkinsky *et al.* 1983). An increase in switching to IgE would result in a greater number of cells expressing IgE, conversely, if fluticasone increases the level of productive epsilon transcription, translation, secretion, or a change in the membrane to secreted IgE ratio, the number of IgE expressing cells would be unchanged.

Differences in the stage at which glucocorticoid is required, and the dependence on monocytes for purified B cell cultures compared to PBMC, have suggested that glucocorticoids have a different mechanism of action in the two cell populations. Though, the requirement of glucocorticoid at the onset of culture for switching to IgE in purified B cell cultures (Jabara *et al.* 1993), and not until day 6 in PBMC (Wu *et al.* 1991), could simply reflect the absence of differentiation signals in purified B cell populations which are necessary for switching, and are provided by accessory cells present in PBMC. In purified B cell cultures, glucocorticoids may provide differentiation signals which replace the need for the accessory cells present in PBMC. The difference in the requirement for monocytes between purified B cell cultures and PBMC could reflect differences in experimental technique, as monocytes are difficult to separate from other cell types. Hence, it is possible that monocytes were present in the purified B cell cultures of Jabara *et al* (1993).

The requirement of monocytes for enhancement of IgE secretion by PBMC suggests that cytokines which inhibit IL-4 induced IgE secretion are produced by monocytes or macrophages. IL-12 and IFN-γ, produced by macrophages are down-regulated by glucocorticoids, and could be a mechanism for IgE enhancement by fluticasone. Although IL-4, added exogenously during the glucocorticoid potentiation experiments,

also favours the development of a TH1 response, inhibiting the production of both IFN-γ from T cells and macrophages and IL-12 from macrophages (Belardelli 1995; Romagnani 1995; Trinchieri 1995; de Waal Malefyt 1997), fluticasone could inhibit levels of these cytokines at later times in culture, when the IL-4 has degraded, and account for the late enhancement of IgE secretion. Studies neutralising antibodies against IL-12 and IFN-γ could help to explore this possibility.

The potentiation of IgE synthesis could also occur through an effect on the efficiency of translation or secretion. Interleukin-6 is well known to increase immunoglobulin secretion, though dexamethasone causes a suppression of the activity of IL-6 on secretion (Shiao *et al.* 1996), suggesting that IL-6 is probably not involved in the glucocorticoid potentiation of IgE secretion. A post-transcriptional mechanism would not explain the ability of glucocorticoids to act as a co-stimulus for switching to IgE in purified B cells, unless this activity is through a different mechanism than for its potentiation of IgE secretion in PBMC.

Glucocorticoids are known to down-regulate surface CD23 expression on B cells, monocytes and eosinophils. As membrane CD23 on B cells mediates a negative feedback loop for IgE synthesis, a down regulation of surface CD23 by fluticasone could enhance IgE levels. Inhibition of the negative feedback loop could occur at a stage after heavy chain switching to IgE through an effect on B cell differentiation, or directly on the efficiency of translation or immunoglobulin secretion. To determine if glucocorticoids act by interfering with the negative feedback route, inhibitors of the CD23-IgE interaction could be used to mimic the effect of glucocorticoid addition.

7.4 CONCLUSIONS

The absence of an effect at day 4.5 on ε germline and VDJ-ε transcript levels by fluticasone indicates that potentiation of IgE synthesis does not occur by an enhancement of switch recombination through the well characterised IL-4 and CD40 induced pathways. Glucocorticoids may act to potentiate IL-4 dependent IgE secretion by acting as a co-stimulus for heavy chain switching through a mechanism other than the IL-4 and CD40 induced pathways at stages later than were tested on day 4.5. Analysis of ε-

germline and VDJ-Cε transcription at later time points could reveal this possibility. The discovery that an enhancement of IgE synthesis does not occur until after 10 days of culture suggests that glucocorticoids could act through a novel mechanism for the up-regulation of IgE synthesis. Further work could reveal that the glucocorticoid receptor has an effect on the stabilisation of an accessible chromatin structure in the switch region or variable region promoter past day 4.5, through an enhanced recruitment of acetyltransferase proteins to the epsilon locus.

The use of an enzyme-linked immunospot assay can help to determine whether the effects of fluticasone occur though an up-regulation of switching. Glucocorticoids may also act through inhibition of a negative feedback loop by the down-regulation of CD23 expression. Neutralising antibodies against IL-12 and IFN-γ, could help to determine if the down-regulation of these cytokines on macrophages by glucocorticoids is responsible for the potentiation of IgE synthesis. The effect of fluticasone could also be mediated through the involvement of the glucocorticoid receptor in the regulation one of the many genes it controls, as well as through the direct inhibition of transcription factors involved in immune function such as NF-κB or AP-1.

Several possibilities could explain the mechanism for glucocorticoid potentiation of IgE secretion. The mechanism could cause increases in germline and productive mRNAs at later time points than were examined in this chapter, or the increases could be through an effect on translation or immunoglobulin secretion.

CONCLUSIONS

IgE and its high affinity receptor perform a central role in the regulation of the allergic response. Consequently, a knowledge of the regulation of both genes will aid in an understanding of the development of atopy and aid in the design of new strategies against allergy.

The gene for the high affinity receptor alpha chain was cloned, and a polymorphism was found in the upstream region sequences. Analysis of the genomic DNA of 30 individuals revealed a potential link between the polymorphism and allergy. An analysis of a larger sample size will be required to confirm that the polymorphism is linked to allergy. The mutation in the promoter region could either increase the chance of developing atopy, through an increase in receptor levels, or it could cause an greater severity of allergic symptoms in an individual. If the link to allergy is proven for this polymorphism, factors that bind to this site should be studied for their role in the regulation of FcεRIα and also for the development of new anti-allergy strategies. Several strong homologies to known transcription factor binding sites were discovered in the putative FcεRIα promoter element, including two strong E boxes, and a two GATA sites, including one potential GATA site that overlaps with the polymorphism. An analysis of the potential role of these sites in regulation of the FcεRIα gene will be important to understand the factors that regulate the allergic response, and to help reveal other key markers linked to atopy.

The sequence of the heavy chain switch recombination junction of the IgE secreting plasmacytoma line U266 revealed a successive switching from IgM→IgA1→IgE. The significance of a prior switching event to α1 for allergy is not known. IgA has not been shown to be involved in the allergic response, but the expression of IgA on the mucus epithelium of the digestive and respiratory tracts is correlates with the location that allergen is encountered and raises the possibility of localised switching to IgE. Further work should concentrate on the possibility that a successive series of switching events from IgM→IgA1→IgE occurs in the local environment of the nasal or bronchial epithelium.

Recombination between μ and α1 occurred at short areas of homology between the respective switch regions in U266. Subsequent reports have indicated that recombination between Sμ and Sε also normally occurs at short regions of homology, supporting the theory that homology between switch regions may have a role in the mechanism of switch recombination.

In each case recombination occurred at regions of repeating pentameric units that characterise switch region topology. The pentameric units were found to occur in a strand specific manner, suggesting a role for these elements in the mechanism of switch recombination, possibly for the alignment of two switch regions prior to recombination. Further insights into the role of switch region structure in the mechanism of switch recombination could allow a better understanding of a potentially unique recombination system, and aid in the design of inhibitors of switching to IgE.

As it is potentially important to the mechanism of switch recombination, the ε-germline gene transcript was cloned, and its regulation was investigated. Expression of the GL-ε transcript preceded the appearance of IgE in IL-4 induced peripheral blood mononuclear cells, supporting the theory that germline transcription precedes switch recombination. The B cell specific factor BSAP/PAX-5 was demonstrated to mediate both IL-4 and CD40 dependent up-regulation of ε-germline gene transcription. As the only B cell specific factor known to bind the GL-ε promoter, BSAP/PAX-5 is potentially responsible for the B cell restricted expression of the ε–germline gene transcript. BSAP is also involved in the regulation of the 3' immunoglobulin locus control region, and the regulation of at least one other germline gene. BSAP is known to participate in several protein-protein interactions, and has therefore been shown to be a candidate to mediate communication between the 3' LCR and the GL-ε promoter. Further work on the role of BSAP in the regulation of the tissue specificity of ε–germline gene transcription could help to elucidate the regulation of the immunoglobulin heavy chain locus. The identification of BSAP as potentially responsible for the tissue specificity of GL-ε transcription could help to target a key event in switching to IgE for anti-allergy strategies.

Further work on the role of the ε-GLT, and the structure of the switch region in the mechanism of switching could also help to clarify the role of transcription and chromatin structure in recombination.

The mechanism of the potentiation of IL-4 induced IgE synthesis by glucocorticoids in PBMC was investigated. The enhancement of IgE secretion by glucocorticoid was found to occur after 10 days of culture. Surprisingly, no effect on germline gene or productive epsilon transcription was found at day 4.5, as was expected from previous work on purified B cells. The lack of an effect at day 4.5 demonstrates that the mechanism of the enhancement of IgE synthesis could occur by a different mechanism than that mediating IL-4/CD40 stimulation of switching to IgE, acting later in the culture period, possibly through an effect on B cell differentiation. An analysis germline and productive epsilon transcription at later times after

the addition of IL-4 could reveal a later acting mechanism for inducing switching to IgE by glucocorticoids than by IL-4/CD40 stimulation. Further work on the glucocorticoid enhancement of IL-4 induced IgE secretion could reveal a novel co-stimulus for switching to IgE.

As glucocorticoids are known to down-regulate CD23 expression, and CD23 has been shown to mediate the induction of apoptosis in B cells, the down-regulation of CD23 by glucocorticoids could lead to the reduction of CD23 induced B cell apoptosis, and therefore an increase in IgE accumulation in culture due to the extended survival of IgE producing cells. CD23 also mediates the negative regulation of IgE synthesis and its down-regulation by glucocorticoids could cause an increase in IgE synthesis by the inhibition of this negative control route. The potential role of CD23 down-regulation in the potentiation of IgE synthesis requires further investigation.

The potential involvement of the JNK signalling cascade in the mechanism of glucocorticoid enhancement of IgE secretion is intriguing. CD40 and EBV both signal through the JNK cascade, and corticosteroid appears to co-stimulate IgE synthesis with IL-4 in purified B cells replacing the need for a co-stimulus from EBV or CD40. Surprisingly, glucocorticoids have been demonstrated to block induction of the JNK cascade. Also, the down-regulation of FcεRII by glucocorticoids is blocked by EBV and CD40 engagement. The effect of CD40 engagement, EBV, and inhibitors of the JNK cascade need to be tested for their effect on the glucocorticoid enhancement of IgE synthesis.

The potential role of histone acetylation of the immunoglobulin locus in the mechanism of the glucocorticoid up-regulation of IgE synthesis requires investigation and could help to explain a novel IgE regulatory route.

Through the discovery of a polymorphism in the FcεRIα promoter region and the identification of a factor, BSAP, potentially responsible for the tissue specific regulation of ε-germline transcription, two new targets for anti-allergy strategies have been proposed. The advances to the regulation of the FcεRIα and the germline ε genes described in this thesis have helped to elucidate two important control points in the regulation of IgE levels. The demonstration of late potentiation of IgE synthesis by glucocorticoids, and a IgM→IgA1→IgE localised switching programme have proposed two novel mechanisms by which IgE levels are regulated.

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Sμ    CTGGGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCAAGGCTAGGCTGAGCTGGGCTGAGCTGAGCTGGG 3921
      ||| ||                          ||||||  |  ||||  |||||||||  ||| ||||||  ||
Sμ    CTGAGCCTAAGCTGAGCCTAGACTAACAGGCTGAACTGGGCTGAGCTGAGCTGAACTGGGCTGAGTTGAA 3668

Sμ    CTGAGCAAGGCTAGGCTGAGCTGAGCTGAGCTGGGCTGCGCTGAGCTGGGCTGGGCTGCGCTGAGCTGGG 3991
      |||  |    |||  ||||||||||||||| |||  | |||  |  || || ||||  ||||  ||||  ||| ||
Sμ    CTGGGTTGAGCTGAGCTGAGCTGAGCTGGGCTAAGTTGCACC-AGGTGAGCTGAGCTGAGCTGGGCTTGG 3738

Sμ    CTGGGCTGAGCTGGGCTAGGCTGGGCTGAGCTGGGCTGAGCTAGGCTGGGCTGGGCTGGGCTGAGCGGGG 4061
      |||  || |||||||||  |||||||  | ||||||||||||  |||||||||||||| ||||  ||  |||
Sμ    CTGCACTAAGCTGGGCTGAGCTGGGCAGGGCTGGGCTGAGCTGAGCTGGGCTGGGCTGAGCTGGGCTGGG 3808

Sμ    CTGAGCGGG-CTGAGCTGAGCTAGGCTGGGCTGAGCGGGG 4100
      ||| || ||  |||||| |      |    |    | |  |
Sμ    CTGGGCTGGGCTGAGCGGTCTAGCGGGCTGAGCTGAGCTG 3848
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Appendix 1. Homology within the μ switch region. Sμ sequences are aligned to show homologous pairing leading to the proposed Sμ deletion event from nucleotides 3781-4033. Sequence of the Sμ regions with identity to the U266 switch recombination join is shown in red.

```
Sμ   ATGGACTCAGATGGCTAAACTGAGCCTAAGCTGAGCCTAGACTAACAGGCTGAACTGGGCTGAGCTGAGC 3650
     || | | | || |           |              |||||||  || ||||||| ||
Sμ   TGATTTGGGCTGGGTTGAGCAGACCTGGGCTGAGCCGGGTTGAGCTGAGCTGAACCAGGATGAGCTGGGC 5290

Sμ   TGAACTGGGCTGAGTTGAACTGGGTTGAGCTGAGCTGAGCTGAGCTGGGCTAAGTTGCACCAGG-TGAGC 3719
     ||| ||| |||| | ||    |    |    |          || | ||        | |    | || ||
Sμ   TGAGCTGAGCTGGGCTGGGTGGTCCAGGCTGGGCTGACCTGGACCAGGCTGGGCCAGGATTGAGCTGGGC 5360

Sμ   TGAGCTGAGCTGGGCTTGGCTGCACTAAGCTGGGCTGAGCTGGGCAGGGCTGGGCTGAGCTGAGCTGGGC 3789
     | ||| |||||| |||  ||||  ||  | ||  ||| ||||||| |||||||||| |||||| ||||||
Sμ   TAAGCCGAGCTGAGCTGAGCTGAGCTGGGATGATCTGGGCTGGGCTGGGCTGGGCTAAGCTGACCTGGGC 5430

Sμ   TGGGCTGAGCTGGGCTGGGCTGGGCTGGGCTGAGCGGTCTAGCGGGCTGAGCTGAGCTGAGGCTGGGCTG 3859
     ||||||| |||  |||| |||| |||| | | |||      | |  |||| ||| ||    |||| |||
Sμ   TGGGCTGGGCTAAGCTGAGCTGAGCTGAGTTAAGCTAAGCTGAG--CTGAACTGGGCCTGTGCTGAGCTA 5498

Sμ   GGCTGAGCTGGGCTGAGCTGGGCTGAGCAAGGCTAGGCTGAGCTGGGCTGAGCTGAGCTGGGCTGAGCAA 3929
     ||||| |||||||||||||||||| ||  ||||  ||     || ||   ||||| ||| |||| || |
Sμ   GGCTGGGCTGGGCTGAGCTGGGCTGGGCTGGGCTGAGCCAGATTGTGCCTGGCTGAACTGAGCTGGGCTA 5568

Sμ   GGCTAGGCTGAGCTGAGCTGAGCTGGGCTGCGCTGAGCTGGGCTGGGCTGCGCTGAGCTGGGCTGGGCTG 3999
     |||  |||| ||||||||| |||| |||| |||| |||| |  |||| | ||||||||| || || |||
Sμ   AGCTGAGCTGGGCTGAGCTGGGCTGAGCTGAGCTGGGCTGAGTGGGGCGGGGCTGAGCTGAGCCGGACTG 5638

Sμ   AGCTGGGCTAGGCTGGGCTGAGCTGGGCTGAGCTAGGCTGGGCTGGGCTGGGCTGAGCGGGGCTGAGCGG 4069
     |||||||| ||||  |||||||  ||||| || ||||||||||  |||||||| || | ||||||| |
Sμ   GGCTGGGCTGGGCTCAGCTGAGCTAAGCTGAACTGGGCTGGGCTGAACTGGGCTGGGCTGAGCTGAGCTG 5708

Sμ   G-CTGAGCTGAGCTAGGCTGGGCTGAGCGGGGCTGAGCTGAGCTAGGCTGGGCTGGGCTGGGCTGAGCCA 4138
     ||| |||| |||   |||||||| || | |||||||| | |  ||||||||  |||||||| |
Sμ   AACTGGGCTGGGCTGAACTGGGCTGGGCTGAGCTGAGCTTGGATGAGCTGGGCTGAACTGGGCTGGGTTG 5778

Sμ   AGCTGAACCGGGTTGAGC-GTGCTGTGCTGGGCTGAGCCAAGCTAGGCTGAGCTGAGCCAAGTTGAGCTT 4207
     |||||  | ||| ||||  | ||    |||  ||| ||  |||   |||| | | |||| || || | |
Sμ   AGCTGGGCTGGGCTGAGTTGAGCCAGACTGATCTGGGCTGAGCCGAGCTGGGTTAAGCCGAGCTGGGTTG 5848

Sμ   AGCTGGGCTGAGCTAACCTGGGCAGGGCTGAGCTGGGCTGAGCTAACCTGGACTGGGCTGAGCTAACCTG 4277
     |||||||| | |   |||||| | || |  ||||| || ||| | |||  |||| ||| ||| | |||
Sμ   GGCTGGGCTGGGTTGGGCTGGGCTGAGCCGGACTGGGTTGGGCTGAGCTGAGCTGGACTGGGCTGAGCTG 5918

Sμ   GGCAGAGCTGAGCTGGGCTGAGCTAACCTGGGCTGGGCTGAGCTAACCTGGGCTGGCGTCAGCTGAGCTG 4347
     || |  ||| ||||| ||||||| | |||   | |     ||||  |||||||||  | |||||  ||||
Sμ   AGCTGGTCTGGGCTGG-CTGAGCTGAGCTGTAGTTGAAGCCGCTAGGCTGGGCTGGGCTGAGCTGGGCTG 5987

Sμ   ACGTACGCTGGGCTGGGCTGGGCTGAGCCGAGCTGAACTGGGCTGAGCAGGCTGTGTCGGGCTGAGCCAA 4417
     |  |  |||| || |   || ||||||| | ||||   || || |||| ||  |    |    |
Sμ   AACTGGGCTGAGCGGACGTGAGCTGAGCTGGGCTGGGTTGAGCAGAGCTGGGGGCTGAGCTGGGCTGGGC 6057

Sμ   GCTGGGCCGAGCTCA 4432
     |   |
Sμ   TGGGCTGAGTTAAGC 6072
```

Appendix 2. Homology within the μ switch region. Sμ sequences are aligned to show homologous pairing leading to the proposed Sμ deletion event from nucleotides 4220-5860. Sequence of the Sμ regions with identity to the U266 switch recombination join is shown in red.

```
Sμ    CATGACAACTCCATCCAGCTTTCAGAAATGGACTCAGATGGCTAAACTGAGCCTAAGCTGAGCCTAGACT 3623
Sα1   GCTGTGCTGTGCTGAGCTGGGCTGGGCTGAGCTGAGCTGGGCTGGGCTGAGCTGAGCTGGGCTGGACTGG 660

Sμ    AACAGGCTGAACTGGGCTGAGCTGAGCTGAACTGGGCTGAGTTGAACTGGGTTGAGCTGAGCTGAGCTGA 3693
Sα1   GCTGGGATGAGCTGGGCTGGGCTGGGCTGAGCTGGGCTGAGCTGGGCTGGGCTGAGCTGAGCTGAGCTGG 730

Sμ    GCTGGGCTAAGTTGCACCAGG-TGAGCTGAGCTGAGCTGGGCTTGGCTGCACTAAGCTGGGCTGAGCTGG 3762
Sα1   GCTGGGCTGGGATGAGCTGGGCTGGGCTGGGCTGGGCTGGGCTGGGCTGGGCTGGGTTGGGCTGAGCTAG 800

Sμ    GCAGGGCTGGGCTGAGCTGAGCTGGGCTGGGCTGAGCTGGGCTGGGCTGGGCTGGGCTGAGCGGTCTAGC 3832
Sα1   ACTGGGCTGAGCTGAGCTGGGCTGAGCTGGACTGGGCTGGGCTGAGCTGGGCCGGGGCGGATTGGGCCGG 870

Sμ    GGGCTGAGCTGAGCTGAGGCTGGGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCAAGGCTAGGCTGAGC 3902
Sα1   GCCCGGGCGGACTGGGCGGGCCGAGCGGGCCGGGGCGGGCTGAGCGGGTCTAGACTGGGCTGAGCTGAGC 940

Sμ    TGGGCTGAGCTGAGCTGGGCTGAGCAAGGCTAGGCTGAGCTGAGCTGAGCTGGGCTGCGCTGAGCTGGGC 3972
Sα1   TGGGNTGGGCTGGGCTTGGACGAGCTGGGCTGCCCTGGGTTGAGCTGAGCTGAGCTGAGCTGGGCTGGCC 1010

Sμ    TGGGCTGCGCTGAGCTGGGCTGGGCTGAGCTGGGCTAGGCTGGGCTGAGCTGGGCTGAGCTAGGCTGGGC 4042
Sα1   TGAGCTGGGCTGGGCTAGGCTGGGCTGGGCTGGGCTGGGCTTGGACGAGCTGGGCTGCCCTGGGTTGAGC 1080

Sμ    TGGGCTGGGCTGAGCGGGGCTGAGCGGGCTGAGCTGAGCTAGGCTGGGCTGAGCGGGGCTGAGCTGAGCT 4112
Sα1   TGAGCTGAGCTGAGCTGGGCTGGGTGAGCTGGGCTGGGCTGGGTTGGGCTGAGCTAGACTGGGCTGGGCT 1150

Sμ    AGGCTGGGCTGGGCTGGGCTGAGCCAAGCTGAACCGGGTTGAGC-GTGCTGTGCTGGGCTGAGCCAAGCT 4181
Sα1   GGGCTGAGCTGGGCTAAGCTGGACTGGGCTGGGCTGGGTTGGGCTGAACTGGGCTGGGCTGAGTTGGGCT 1220

                  deletion, nt 4220-5860
                                  ↓
Sμ    AGGCTGAGCTGAGCCAAGTTGAGCTTAGCTGGGCTGAGTTGGGCTGGGCTGAGCCGGACTGGGTTGGGCT 5892
Sα1   GAGCTGGGCTGAGCTGAGTTGGGCTGAGCTGGGCTGAGCTGAGTTGGGCTGAGCTGGGCTAAGCTGGACC 1290

Sμ    GAGCTGAGCTGGACTGGGCTGAGCTGAGCTGGTCTGGGCTGG-CTGAGCTGAGCTGTAGTTGAAGCCGCT 5961
Sα1   TGGCTGGGGTGAGCTGGGCTGGGCTGCGCTGAGCTGGGCTGGGCTGGGCTGGGCTGAGCTGGGCTGGGCT 1360

Sμ    AGGCTGGGCTGGGCTGAGCTGGGCTGAACTGGGCTGAGCGGACGTGAGCTGAGCTGGGCTGGGTTGAGCA 6031
Sα1   GAGCTGAGCTGGGCTGACCTGGGCTGGGCTGACCTGGGCTGGGCTAAGCTGGACTGATCTGGGCTAGGCT 1430

Sμ    GAGCTGGGGGCTGAGCTGGGCTGGGCTGGGCTGAGTTAAGCTGGGCTGACCTGGGCTGAGTTAAGCTGGG 6101
Sα1   GGGCTGGG--CTGACCTAAGCTGAGCCAGACTGGGCTGAGCTGGGTGAGCTTAGGTGGATTGAGCCTGGA 1498

Sμ    CTGACCTGGGCTGAGCTAAGCTGGGTTGAGCCGATCCGGGCTGGGCTGGGCTGGGCTGAGCCAGGCTAGG 6171
Sα1   CTGGGCTGAGCTGAGCTGGGCTGAGCTGGACTGGGCTAGGCTGAGCTGAGCTTGGTTGGGTTGACCTGGG 1568
```

```
Sμ    TTGAGCTGGGCTGGGCTGGGCTCTGCTGTGCTGTGCTGAACAGGGCTGAGCTGAACTGAGCTGAGCTGGG 6241
      ||||||| ||||   ||||  |   ||| |||| ||||     ||||| |||   |||| ||| ||||||
Sα1   CTGAGCTGAGCTGAATTGGGTTGACCTGGGCTGAGCTGGGTTCGGCTGGGCTAGGCTGACCTGGGCTGGG 1638

Sμ    CTGAGCTG-GCTCTGCTGTGCTGTGCTGAGCTAGGGCTGAGCTGAAGCGTGCTGAGCTGGGTTGAGCTAA 6310
      |||||||| |||  |||  | || |||||||| |||||| | || |  | |||| |||||||||  ||
Sα1   CTGAGCTGAGCTAGGCTTGGATGAGCTGAGCT-GGGCTGGGTTGGACTGCGCTGGGCTGGGTTGGACTGG 1707

Sμ    GCTAGGCTGGGCTGGGCTGAGCTGGGATGAGCTGGGCTGGGCTGGGCTGGGCTCTGCTGTGCTGTGCTGA 6380
      || |||||  ||| ||||  ||| | ||  |||  ||| |||| ||||||||  |||  |||| ||||
Sα1   TCTGGGCTGACCTGAGCTGTACTGAGCTGGTCTGACCTGTGCTGAGCTGGGCTGGGCTAGGCTGAGCTGG 1777

Sμ    ACAGGGCTGAGCTGAACTGAGCTGAGCTGGGCTGAGCTGAGCTCTGCTGTGCTGTGCTGAGCAGGGCTGA 6450
       | |  ||| | ||  ||| |||| |||  |||| |||  |||  |||| ||||  | |||| |  |||
Sα1   GCTGACCTGTGTTGGGCTGGGCTGGGCTAAGCTGGGCTAGGCTGGGCTGAGCTGGACGGAGCTGACCTGG 1847

Sμ    GCTGAACTGGGCTGAGCTGGGCTGAGCCGGGCTGAGTTGAGCAGAGCTGGGTTGAGCAGAGCTGGGCTGG 6520
      | ||| ||    ||  ||| ||||||| || ||||  || ||  ||||  | || || || ||| |||||
Sα1   G-TGAGCTTAAGTGGACTGAGCTGAGCTGGACTGACCTGGGCTAAGCTAAGCTGGGCTGACCTGTGCTGG 1916

Sμ    GCTGGGCTGAGTTGAGCCAGGCTGACCTGGGCTGAGCCAAGCTGGGTTGAGCCAAACTGGGCTGGGCTGG 6590
      |||||||||   | ||  |||||| |||| ||||||  |||| |  |||||  | |||||  || ||||
Sα1   GCTGGGCTGGACTAGGCTGGGCTGAGCTGGACTGAGCTGAGCTAGACTGAGCTGAGCTGGG-TGAGCTGA 1985

Sμ    GCTGAGCCAGGCTGGATTGAGCTGGGCTGGGCTGGGCTAAGCTGGGCTGTGCTGCACTGAGCTGGGCTGA 6660
      | ||  |   |||||  || |||| |||| ||||| ||  |||||||    |||  ||   |||||||||
Sα1   GGTGGACTGAGCTGGGCTGGGCTGAGCTGAGCTGGACTGGGCTGGGCGAA-CTGAGCTAGACTGGGCTGA 2054

Sμ    GCTGGGTTGAACTGTGCTGAGCTGAGCTGGGCTGTGCTGAACTATGCTTAGCTGGGCTGGGCTATACTGG 6730
      ||||||  |||  |||  | ||  |||||||| |||||  ||||   |  |||  |||||||||| ||||  |||
Sα1   GCTGGGCTGAGCTGGGGTGGGCTGAGCTAGGCTGAGCTGGGGTGGGCTGAGCTGGGCTGAGCTAGGCTGA 2124

Sμ    GCTTAGCTGGGCTGGGCTAAACTGGGCTTAGCTGGGCTGGGCTATACTGGGCTTAGCTGGGCTGGGCTAT 6800
      |||  |||||||  | ||  | |  |||  ||  |||||  | ||  |||   ||  ||||  ||||  ||||||||
Sα1   GCTGAGCTGGGTTTGGTTGACATGGACTGAGCTGTGTTGAGCTGGGCTAGGCTAAGCTAGGCTGGGCGGG 2194

Sμ    ACTGGGCTTAGCTGGGCTGGGCTATACTGGGCTTAGCTGGGCTGGGCTGAGCTGAGATGGTCTTAGGTGG 6870
        ||  |||   |  |||||||  |||  ||||||||   |||||||| |||  ||||  |  |||  ||   |  |||
Sα1   G-TGAGCTGGGTTGGGCTGAGCTGAACTGGGCTGGACTGGGCTGGACTGGGCTGGGCTGGGCTGGGCTGG 2263

Sμ    TCTGAGCTCATCTGAGTTGGGCTGAGCTGAGCTTGGCTTTGCTGAGCTGGGGTGGGGTGGGCTGGGCTGG 6940
       |||||||  |  |||||  ||  ||||||||  ||  |||   |||||||||||  ||||  ||  ||||  ||
Sα1   GCTGAGCTGAGCTGAGCTGTACTGAGCTGGCCTGGGCGA-GCTGAGCTGGGCTGGGCTGAGCTGTACTAA 2332

Sμ    ATTGAGCTGGCCTGGGCTGGGATGAACTGGAGGACATGGCACTGGGCCAATCTTCATGATCTTGTTGGAC 7010
        ||  ||||  ||||    |    |    |  | | |||    ||   |       |||  ||  |    ||
Sα1   GCTGGCCTGGGCTGGCTGAGCTGTACTGAGCTGGCCTGGGCTGGGTTGACCTGGGCTGAGCTGGACTAAC 2402
```

Appendix 3. Homology between the μ and α1 switch regions. Sμ and Sα1 sequences are aligned to show homologous pairing leading to the proposed μ/α1 switch recombination event. Sequence of the Sμ and Sα1 regions with identity to the U266 switch recombination join is shown in red.

```
Sα1  GGCTTGGACGAGCTGGGCTGCCCTGGGTTGAGCTGAGCTGAGCTGAGCTGGGCTGGGTGAGCTGGGCTGG 1117
Sε   AAACTGGACCGGGCTAAATTGATCTGGACTGACCATTCTCACCTGGCTAAGAGGAGCTGAGTCAGAAGCA 2998
Sμ   GCTCAGCTGAGCTAAGCTGAACTGGGCTGGGCTGAACTGGGCTGGGCTGAGCTGAGCTGAACTGGGCTGG 5719

Sα1  GCTGGGTTGGGCTGAGCTAGACTGGGCTGGGCTGGGCTGAGCTGGGCTAAGCTGGACTGGGCTGGGCTGG 1187
Sε   AGCTGGTTGAGCTGGCTGGACTGAAATAAGAGTTTGCTGCCTGCAAGGGGAGGTCCTGGGCTGACCTGGG 3068
Sμ   GCTGAACTGGGCTGGGCTGAGCTGAGCTTGGATGAGCTGGGCTGAACTGGGCTGGGTTGAGCTGGGCTGG 5789

Sα1  GTTGGGCTGAACTGGGCTGGGCTGAGTTGGGCTGAGCTGGGCTGAGCTGAGTTGGGCTGAGCTGGGCTGA 1257
Sε   CCAGGCTGAACCAGGCTGGCTTAGAGTGAACTTCAGAGGGCGACTCCCCCGGTAGGCCAGTCTCAGCTGA 3138
Sμ   GCTGAGTTGAGCCAGACTGATCTGGGCTGAGCCGAGCTGGGTTAAGCCGAGCTGGGTTGGGCTGGGCTGG 5859

Sα1  GCTGAGTTGGGCTGAGCTGGGCTAAGCTGGACCTGGCTGGGGTGAGCTGGGCTGGGCTGCGCTGAGCTGG 1237
Sε   ACTTGGCTGTCCCGGTGGGCAGAGCGGGGCTGGATACTGTGATTTTGGGGGTACCTAGAGCAGACTTCAA 3208
Sμ   GTTGGGCTGGGCTGAGCCGGACTGGGTTGGGCTGAGCTGAGCTGGACTGGGCTGAGCTGAGCTGGTCTGG 5929

Sα1  GCTGGGCTGGGCTGGGCTGAGCTGGGCTGGGCTGAGCTGAGCTGGGCTGACCTGGGCTGGGCTGACCTGG 1397
Sε   GACCAAGCTAAACTGGGCTCCAGGGGCAGGATGGGCTGGGGACTTGGGACTCCAGGCCAGGGGCGAAGGG 3278
Sμ   GCTGGCTGAGCTGAGCTGTAGTTGAAGCCGCTAGGCTGGGCTGGGCTGAGCTGGGCTGAACTGGGCTGAG 5999

Sα1  GCTGGGCTAAGCTGGACTGATCTGGGCTAGGCTGGGCTGGGCTGACCTAAGCTGAGCCAGACTGGGCTGA 1467
Sε   CCACGCTGTACAGACCGCACTATCTGGGCCAGGGTTCTGTGGTGGGAGGGACTGACTGCCTGGGGCATCA 3348
Sμ   CGGACGTGAGCTGAGCTGGGCTGGGTTGAGCAGAGCTGGGGGCTGAGCTGGGCTGGGCTGGGCTGAGTTA 6069

Sα1  GCTGGGTGAGCTTAGGTGGATTGAGCCTGGACTGGGCTGAGCTGAGCTGGGCTGAGCTGGACTGGGCTAG 1537
Sε   GGGCAAGTCTTCCCGCCCTCCCCTAGAGGTCAGGGGTGGGCAGAGCACCATGGGGGTCTGGCAGGTCAGG 3418
Sμ   AGCTGGGCTGACCTGGGCTGAGTTAAGCTGGGCTGACCTGGGCTGAGCTAAGCTGGGTTGAGCCGATCCG 6139

Sα1  GCTGAGCTGAGCTTGGTTGGGTTGACCTGGGCTGAGCTGAGCTGAATTGGGTTGACCTGGGCTGAGCTGG 1607
Sε   TGAGGGCTGCTGTGATGGGGAGATCCAGGCTTGGCACTCAAGAGCCCGAGGAGCTGAGACCACAGCCTTG 3488
Sμ   GGCTGGGCTGGGCTGGGCTGAGCCAGGCTAGGTTGAGCTGGGCTGGGCTGGGCTCTGCTGTGCTGTGCTG 6209
```

Appendix 4. Homology between Sε and the Sα1 and Sμ regions. The epsilon switch region (Sε) is compared to the alpha 1 (Sα1) and mu (Sμ) switch regions. Identity with the U266 sequence is in red.

```
   1 AGCACGGGTCTCTCTGTGGCCAGGGCCACCCTAGGCCTCTGGGGTCCAATGCCCAACAACCCCCGGGCCC
  71 TCCCCGGGCTCAGTCTGAGAGGGTCCCAGGGACGTAGCGGGGCGCCAGTTCTTGCCTGGGGTCCTGGCAT
 141 TGTTGTCACAATGTGACAACTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGTGA
 211 GTCCTCACCACCCCCTCTCTGAGTCCACTTAGGGAGACTCAGCTTGCCAGGGTCTCAGGGTCAGAGTCTT
 281 GGAGGCATTTTGGAGGTCAGGAAAGAAAGCCGGGGAGAGGGACCCTTCGAATGGGAACCCAGCCTGTCCT
 351 CCCCAAGTCCGGCCACAGATGTCGGCAGCTGGGGGGCTCCTTCGGCTGGTCTGGGGTGACCTCTCTCCGC
 421 TTCACCTGGAGCATTCTCAGGGGCTGTCGTGATGATTGCGTGGTGGGACTCTGTCCCGCTCCAAGGCACC
 491 CGCTCTCTGGGACGGGTGCCCCCCGGGGTTTTTGGACTCCTGGGGGTGACTTAGCAGCCGTCTGCTTGCA
 561 GTTGGACTTCCCAGGCCGACAGTGGTCTGGCTTCTGAGGGGTCAGGCCAGAATGTGGGGTACGTGGGAGG
 631 CCAGCAGAGGGTTCCATGAGAAGGGCAGGACAGGGCCACGGACAGTCAGCTTCCATGTGACGCCCGGAGA
 701 CAGAAGGTCTCTGGGTGGCTGGGTTTTTGTGGGGTGAGGATGGACATTCTGCCATTGTGATTACTACTAC
 771 TACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGTAAGAATGGCCACTCTA
 841 GGGCCTTTGTTTTCTGCTACTGCCTGTGGGGTTTCCTGAGCATTGCAGGTTGGTCCTCGGGGCATGTTCC
 911 GAGGGGACCTGGGCGGACTGGCCAGGAGGGGACGGGCACTGGGGTGCCTTGAGGATCTGGGAGCCTCTGT
 981 GGATTTTCCGATGCCTTTGGAAAATGGGACTCAGGTTGGGTGCGTCTGATGGAGTAACTGAGCCTGGGGG
1051 CTTGGGGAGCCACATTTGGACGAGATGCCTGAACAAACCAGGGGTCTTAGTGATGGCTGAGGAATGTGTC
1121 TCAGGAGCGGTGTCTGTAGGACTGCAAGATCGCTGCACAGCAGCGAATCGTGAAATATTTTCTTTAGAAT
1191 TATGAGGTGCGCTGTGTGTCAACCTGCATCTTAAATTCTTTATTGGCTGGAAAGAGAACTGTCGGAGTGG
1261 GTGAATCCAGCCAGGAGGGACGCGTAGCCCCGGTCTTGATGAGAGCAGGGTTGGGGGCAGGGGTAGCCCA
1331 GAAACGGTGGCTGCCGTCCTGACAGGGGCTTAGGGAGGCTCCAGGACCTCAGTGCCTTGAAGCTGGTTTC
1401 CATGAGAAAAGGATTGTTTATCTTAGGAGGCATGCTTACTGTTAAAAGACAGGATATGTTTGAAGTGGCT
1471 TCTGAGAAAAATGGTTAAGAAAATTATGACTTAAAAATGTGAGAGATTTTCAAGTATATTAATTTTTTTA
1541 ACTGTCCAAGTATTTGAAATTCTTATCATTTGATTAACACCCATGAGTGATATGTGTCTGGAATTGAGGC
1611 CAAAGCAAGCTCAGCTAAGAAATACTAGCACAGTGCTGTCGGCCCCGATGCGGGACTGCGTTTTGACCAT
1681 CATAAATCAAGTTTATTTTTTTAATTAATTGAGCGAAGCTGGAAGCAGATGATGAATTAGAGTCAAGATG
1751 GCTGCATGGGGGTCTCCGGCACCCACAGCAGGTGGCAGGAAGCAGGTCACCGCGAGAGTCTATTTTAGGA
1821 AGCAAAAAAACACAATTGGTAAATTTATCACTTCTGGTTGTGAAGAGGTGGTTTTGCCCAGGCCCAGATC
1891 TGAAAGTGCTCTACTGAGCAAAACAACACCTGGACAATTTGCGTTTCTAAAATAAGGCGAGGCTGACCGA
1961 AACTGAAAAGGCTTTTTTTAACTATCTGAATTTCATTTCCAATCTTAGCTTATCAACTGCTAGTTTGTGC
2031 AAACAGCATATCAACTTCTAAACTGCATTCATTTTTAAAGTAAGATGTTTAAGAAATTAAACAGTCTTAG
2101 GGAGAGTTTATGACTGTATTCAAAAAGTTTTTTAAATTAGCTTGTTATCCCTTCATGTGATAATTAATCT
2171 CAAATACTTTTTCGATACCTCAGAGCATTATTTTCATAATGACTGTGTTCACAATCTTTTTAGGTTAACT
2241 CGTTTTCTCTTTGTGATTAAGGAGAAACACTTTGATATTCTGATAGAGTGGCCTTCATTTTAGTATTTTT
2311 CAAGACCACTTTTCAACTACTCACTTTAGGATAAGTTTTAGGTAAAATGTGCATCATTATCCTGAATTAT
2381 TTCAGTTAAGCATGTTAGTTGGTGGCATAAGAGAAAACTCAATCAGATAGTGCTGAAGACAGGACTGTGG
2451 AGACACCTTAGAAGGACAGATTCTGTTCCGAATCACCGATGCGGCGTCAGCAGGACTGGCCTAGCGGAGG
2521 CTCTGGGAGGGTGGCTGCCAGGCCCGGCCTGGGCTTTGGGTCTCCCCGGACTACCCAGAGCTGGGATGCG
2591 TGGCTTCTGCTGCCGGGCCGACTGGCTGCTCAGGCCCCAGCCCTTGTTAATGGACTTGGAGGAATGATTC
2661 CATGCCAAAGCTTTGCAAGGCTCGCAGTGACCAGGCGCCCGACATGGTAAGAGACAGGCAGCCGCCGCTG
2731 CTGCATTTGCTTCTCTTAAAACTTTGTATTTGACGTCTTATTTCCACTAGAAGGGGAACTGGTCTTAATT
2801 GCTTGATGAAGAGCAGGAGACTCATTTATGTGAGTCTTTTGAGTGACCATTGTCTGGGTCACTCCCATTT
2871 AACTTTCCCTAAAGCCCATTTGAAGGAGAGGTCGCACGAGCTGCTCCACAACCTCTGAATGGGGATGGCA
2941 TGGGTAATGATGCTTGAGAACATACCAAGCCCCACTGGCATCGCCCTTGTCTAAGTCATTGACTGTAGGT
3011 CATCATCGCACCCTTGAAAGTAGCCCATGCCTTCCAAAGCGATTTATGGTAAATGGCAGAATTTTAAGTG
3081 GCAAATTCAGATAAAATGCATTTCTTGGTTGTTTCCAATGATGACTGTTATCTAGAGGGAATTTAAAGGC
3151 AGGGGTTTACTGCAGACTCAGAAGGGAGGGGATGCTCCGGGAAGGTGGAGGCTCTGAGCATCTCAATACC
3221 CTCCTCTTGGTGCAGAAGATATGCTGCCACTTCTAGACAAGGGGACCTGCTCATTTTTATCACAGCACAG
3291 GCTCCTAAATTCTTGGTCTCATTCTCAAGATGTTTTAATGACTTTAAAGCAGCAAAGAAATATTCCACCC
3361 AGGTAGTGGAGGGTGGTAATGATTGGTAATGCTTTGGAACCAAAACCCAGGTGGCGCTGGGGCAGGACTG
                                                    SKS287(Sμ/Sε)
                                                          |
3431 CAGGGAACTGGGGTATCAAGTAGAGGGAGACAAAAGATGGAAGCCAGCCTGGCTGTGCAGGAACCCGGCA
3501 ATGAGATGGCTTTAGCTGAGACAAGCAGGGCTGGTGGGCTGACCATTTCTGGCCATGACAACTCCATCCA
                        HJS7-2/SKS285(Sμ/Sε)
                                    | |
3571 GCTTTCAGAAATGGACTCAGATGGCTAAACTGAGCCTAAGCTGAGCCTAGACTAACAGGCTGAACTGGGC
```

SKS288/SKS283/SKS282/SKS286(Sμ/Sε)

3641 TGAGCTGAGCTGAACTGGGCTGAGTTGAACTGGGTTGAGCTGAGCTGAGCTGAGCTGGGCTAAGTTGCAC

SKS281/A5-2(Sμ/Sε)

3711 CAGGTGAGCTGAGCTGAGCTGGGCTTGGCTGCACTAAGCTGGGCTGAGCTGGGCAGGGCTGGGCTGAGCT

HJS7-1(Sμ/Sε) 2C10(Sμ/Sγ4/Sε) pSC1.7-56(Sμ/Sγ4/Sε)

3781 GAGCTGGGCTGGGCTGAGCTGGGCTGGGCTGGGCTGGGCTGAGCGGTCTAGCGGGCTGAGCTGAGCTGAG

HJS6(Sμ/Sε)

3851 GCTGGGCTGGGCTGAGCTGGGCTGAGCTGGGCTGAGCAAGGCTAGGCTGAGCTGGGCTGAGCTGAGCTGG
3921 GCTGAGCAAGGCTAGGCTGAGCTGAGCTGAGCTGGGCTGCGCTGAGCTGGGCTGGGCTGCGCTGAGCTGG

2C4(Sμ/Sε)

3991 GCTGGGCTGAGCTGGGCTAGGCTGGGCTGAGCTGGGCTGAGCTAGGCTGGGCTGGGCTGGGCTGAGCGGG
4061 GCTGAGCGGGCTGAGCTGAGCTAGGCTGGGCTGAGCGGGGCTGAGCTGAGCTAGGCTGGGCTGGGCTGGG
4131 CTGAGCCAAGCTGAACCGGGTTGAGCGTGCTGTGCTGGGCTGAGCCAAGCTAGGCTGAGCTGAGCCAAGT

U266(Sμ/Sε)

4201 TGAGCTTAGCTGGGCTGAGCTAACCTGGGCAGGGCTGAGCTGGGCTGAGCTAACCTGGACTGGGCTGAGC

A5-1(Sμ/Sε)

4271 TAACCTGGGCAGAGCTGAGCTGGGCTGAGCTAACCTGGGCTGGGCTGAGCTAACCTGGGCTGGCGTCAGC
4341 TGAGCTGACGTACGCTGGGCTGGGCTGGGCTGAGCCGAGCTGAACTGGGCTGAGCAGGCTGTGTCGGGCT
4411 GAGCCAAGCTGGGCCGAGCTCAGCAGAGCTGAGGAGCTGAGCTTAGCTGGGCTGAGCTAACCAGGGCTGG
4481 GCTGAGCTGGGCTGAGCTGAGCTGAGCTGAACTGGGCTGAACGGGCTGAGGCGAGCAGGGCCGAGCTGAG
4551 CAGAGCTAAGCCGAGGCTGGGCTGGGCTAACCTGGGCTGGGCGAGCTGAGCAGAGCTAAGCCGAGGCTGG
4621 GCTGGGCTAACCTGGGCTGGGCTGAGCTGAGCTGGGTTGGGCAGGGCTGGGCTGGGCTGAGCTAAGCTGA
4691 ACTAGGGTGAGCTGGGCCGAGCCAGGGCTGGTTTGGGCTGAGTTGAGCTGACCTGGACTGGGCTGAGCTA
4761 ACCTGGGCTGGGCTCAGCTGAGCTACGCTGGGCTGGGCTGGGCTGAGCCGAGCTGAACTGGGCTGAGCAG
4831 GTTGTATCAGGCTAAGCCAAGCTGGGCCAAGCTCAGTAGAGCCGAGCCGAGCTGAGCTTAGCTGGGCTCA
4901 GCTAACCAGGGCTGGGCTGAGCTGGGCTGAGCTGAGCTGAGCTGAACTGGCTGAACGGGCTGAGGCGAGC
4971 AGGGCCGAGCTGAGCAGAGCTAAGCCGAGGCTGGGCTGGGCTAACCTGGGCTGGGCTGAGCTGAGCTGGG
5041 TTGGGCAAGGCTGGGCTGGGTTGAGTTGGGTGAGCTAAGCTGAGCTAGGGTGAGCTGGGCTGAGCCAGGC
5111 TGGATTGGGCTGAGTTGAGCTGACCTCAACTGAGCTGACCTAGGCTGAGCTGAGCTGAGCTGAAGTAGGC
5181 TGTGCTGGGCTGAGCTGGGATGACCTGGGCTGGGCTGAGCTGATTTGGGCTGGGTTGAGCAGACCTGGGC
5251 TGAGCCGGGTTGAGCTGAGCTGAACCAGGATGAGCTGGGCTGAGCTGAGCTGGGCTGGGTGGTCCAGGCT
5321 GGGCTGACCTGGACCAGGCTGGGCCAGGATTGAGCTGGGCTAAGCCGAGCTGAGCTGAGCTGAGCTGGGA
5391 TGATCTGGGCTGGGCTGGGCTGGGCTAAGCTGACCTGGGCTGGGCTGGGCTAAGCTGAGCTGAGCTGAGT
5461 TAAGCTAAGCTGAGCTGAACTGGGCCTGTGCTGAGCTAGGCTGGGCTGGGCTGAGCTGGGCTGGGCTGGG
5531 CTGAGCCAGATTGTGCCTGGCTGAACTGAGCTGGGCTAAGCTGAGCTGGGCTGAGCTGGGCTGAGCTGAG
5601 CTGGGCTGAGTGGGGCGGGGCTGAGCTGAGCCGGACTGGGCTGGGCTGGGCTCAGCTGAGCTAAGCTGAA
5671 CTGGGCTGGGCTGAACTGGGCTGGGCTGAGCTGAGCTGAACTGGGCTGGGCTGAACTGGGCTGGGCTGAG
5741 CTGAGCTTGGATGAGCTGGGCTGAACTGGGCTGGGTTGAGCTGGGCTGGGCTGAGTTGAGCCAGACTGAT
5811 CTGGGCTGAGCCGAGCTGGGTTAAGCCGAGCTGGGTTGGGCTGGGCTGGGTTGGGCTGGGCTGAGCCGGA
5881 CTGGGTTGGGCTGAGCTGAGCTGGACTGGGCTGAGCTGAGCTGGTCTGGGCTGGCTGAGCTGAGCTGTAG

pSC1.8-23(Sμ/Sε)

5951 TTGAAGCCGCTAGGCTGGGCTGGGCTGAGCTGGGCTGAACTGGGCTGAGCGGACGTGAGCTGAGCTGGGC
6021 TGGGTTGAGCAGAGCTGGGGGCTGAGCTGGGCTGGGCTGGGCTGAGTTAAGCTGGGCTGACCTGGGCTGA
6091 GTTAAGCTGGGCTGACCTGGGCTGAGCTAAGCTGGGTTGAGCCGATCCGGGCTGGGCTGGGCTGGGCTGA
6161 GCCAGGCTAGGTTGAGCTGGGCTGGGCTGGGCTCTGCTGTGCTGTGCTGAACAGGGCTGAGCTGAACTGA
6231 GCTGAGCTGGGCTGAGCTGGCTCTGCTGTGCTGTGCTGAGCTAGGGCTGAGCTGAAGCGTGCTGAGCTGG
6301 GTTGAGCTAAGCTAGGCTGGGCTGGGCTGAGCTGGGATGAGCTGGGCTGGGCTGGGCTGGGCTCTGCTGT

pSC1.8-10(Sμ/Sε)

6371 GCTGTGCTGAACAGGGCTGAGCTGAACTGAGCTGAGCTGGGCTGAGCTGAGCTCTGCTGTGCTGTGCTGA
6441 GCAGGGCTGAGCTGAACTGGGCTGAGCTGGGCTGAGCCGGGCTGAGTTGAGCAGAGCTGGGTTGAGCAGA
6511 GCTGGGCTGGGCTGGGCTGAGTTGAGCCAGGCTGACCTGGGCTGAGCCAAGCTGGGTTGAGCCAAACTGG
6581 GCTGGGCTGGGCTGAGCCAGGCTGGATTGAGCTGGGCTGGGCTGGGCTAAGCTGGGCTGTGCTGCACTGA

pSC1.7-4(Sμ/Sε)　　pSC1.1-4(Sμ/Sε)

6651 GCTGGGCTGAGCTGGGTTGAACTGTGCTGAGCTGAGCTGGGCTGTGCTGAACTATGCTTAGCTGGGCTGG

pSC1.7-12(Sμ/Sε)

6721 GCTATACTGGGCTTAGCTGGGCTGGGCTAAACTGGGCTTAGCTGGGCTGGGCTATACTGGGCTTAGCTGG

pSC1.1-7(Sμ/Sε)

6791 GCTGGGCTATACTGGGCTTAGCTGGGCTGGGCTATACTGGGCTTAGCTGGGCTGGGCTGAGCTGAGATGG
6861 TCTTAGGTGGTCTGAGCTCATCTGAGTTGGGCTGAGCTGAGCTTGGCTTTGCTGAGCTGGGGTGGGGTGG

pSC1.7-6/pSC1.1-12/pSC1.7-2/pSC1.7-1(Sμ/Sε)

6931 GCTGGGCTGGATTGAGCTGGCCTGGGCTGGGATGAACTGGAGGACATGGCACTGGGCCAATCTTCATGAT
7001 CTTGTTGGACATAGATGGATAGCCTCAGCTGAGTCTACACTGCGTTCCCCATCACACTCACCCTCCCTAT
7071 ACTCACTCCCAGGCCTGGGTTGTCTGCCTGGGGAGACTTCAGGGTAGCTGGAGTGTGACTGAGCTGGGGG
7141 CAGCAGAAGCTGGGCTGGAGGGACTCTATTGGCTGCCTGCGGGGAGTGTGGCTCCAGGCTTCACATTCAG
7211 GTATGCAACCTGGGCCCTCCAGCTGATGTGTCTGGGAGCTGAGTGTGTGCACGACCTACGTGCTGATGCC
7281 TGGGGGAAAGCAGGCCTGGTCCACCCAAACCTGAGCCCTCAGCCATCTGAGCAGGGAGCCAGGGGCAGTC
7351 AGGCCTCAGAGTGCAGCAGGGCAGCCAGCTGAATGGTGGCAGGGATGGCTCAGCCTGCTCCAGGAGACCC
7421 CAGGTCTGTCCAGGTGTTCAGTCTGGGCCCTGCAGCAGGATGGGCTGAGGCCTGCAGCCCCAGCAGCCTT
7491 GGACAAAGACCTGAGGCCTCACCACGGCCCCGCCACCCCTGATAGCCATGACAGTCTGGGCTTTGGAGGC
7561 CTGCAGGTGGGCTCGGCCTTGGTGGGGCAGCCACAGCGGGACGCAAGTAGTGAGGGCACTCAGAACGCCA
7631 CTCAGCCCTGACAGGCAGGGCACGAGGAGGCAGCTCCTCACCCTCCCTTTCTCTTTTGTCCTGCGGGTCC
7701 TCAGGGAGTGCATCCGCCCCAACCCTTTTCCCCCTCGTCTCCTGTGAGAATTCCCCGTCGGATACGAGCA
7771 GCGTGGCCGTTGGCTGCCTCGCACAGGACTTCCTTCCCGACTCCATCACTTTCTCCTGGAAATACAAGAA
7841 CAACTCTGACATCAGCAGCACCCGGGCTTCCCATCAGTCCTGAGAGGGGGCAAGTACGCAGCCACCTCA
7911 CAGGTGCTGCTGCCTTCCAAGGACGTCATGCAGGGCACAGACGAACACGTGGTGTGCAAAGTCCAGCACC
7981 CCAACGGCAACAAAGAAAAGAACGTGCCTCTTCCAGGTGAGGGCCGGGCCCAGCCACCGGGACAGAGAGG
8051 GAGCCGAAGGGGGCGGGAGTGGCGGGCACCGGGCTGACACGTGTCCCTCACTGCAGTGATTGCTGAGCTG
8121 CCTCCCAAAGTGAGCGTCTTCGTCCCACCCCGCGACGGCTTCTTCGGCAACCCCCGCAAGTCCAAGCTCA
8191 TCTGCCAGGCCACGGGTTTCAGTCCCCGGCAGATTCAGGTGTCCTGGCTGCGCGAGGGGAAGCAGGTGGG
8261 GTCTGGCGTCACCACGGACCAGGTGCAGGCTGAGGCCAAAGAGTCTGGGCCCACGACCTACAAGGTGACC
8331 AGCACACTGACCATCAAAGAGAGCGACTGGCTCAGCCAGAGCATGTTCACCTGCCGCGTGGATCACAGGG
8401 GCCTGACCTTCCAGCAGAATGCGTCCTCCATGTGTGTCCCCGGTGAGTGACCTGTCCCCAGGGGCAGCAC
8471 CCACCGACACACAGGGGTCCACTCGGGTCTGGCATTCGCCACCCCGGATGCAGCCATCTACTCCCTGAGC
8541 CTTGGCTTCCCAGAGCGGCCAAGGGCAGGGGCTCGGGCGGCAGGACCCCTGGGCTCGGCAGAGGCAGTTG
8611 CTACTCTTTGGGTGGGAACCATGCCTCCGCCCACATCCACACCTGCCCCACCTCTGACTCCCTTCTCTTG
8681 ACTCCAGATCAAGACACAGCCATCCGGGTCTTCGCCATCCCCCCATCCTTTGCCAGCATCTTCCTCACCA
8751 AGTCCACCAAGTTGACCTGCCTGGTCACAGACCTGACCACCTATGACAGCGTGACCATCTCCTGGACCCG
8821 CCAGAATGGCGAAGCTGTGAAAACCCACACCAACATCTCCGAGAGCCACCCCAATGCCACTTTCAGCGCC
8891 GTGGGTGAGGCCAGCATCTGCGAGGATGACTGGAATTCCGGGGAGAGGTTCACGTGCACCGTGACCCACA
8961 CAGACCTGCCCTCGCCACTGAAGCAGACCATCTCCCGGCCCAAGGGTAGGCCCCACTCTTGCCCCTCTTC
9031 CTGCACTCCCTGGGACCTCCCTTGGCCTCTGGGGCATGGTGGAAAGCACCCCTCACTCCCCCGTTGTCTG
9101 GGCAACTGGGGAAAAGGGGACTCAACCCCAGCCCACAGGCTGGCCCCCCACTGCCCCGCCCTCACCACCA
9171 TCTCTGTTCACAGGGGTGGCCCTGCACAGGCCCGATGTCTACTTGCTGCCACCAGCCCGGGAGCAGCTGA
9241 ACCTGCGGGAGTCGGCCACCATCACGTGCCTGGTGACGGGCTTCTCTCCCGCGGACGTCTTCGTGCAGTG
9311 GATGCAGAGGGGGCAGCCCTTGTCCCCGGAGAAGTATGTGACCAGCGCCCCAATGCCTGAGCCCCAGGCC
9381 CCAGGCCGGTACTTCGCCCACAGCATCCTGACCGTGTCCGAAGAGGAATGGAACACGGGGGAGACCTACA
9451 CCTGCGTGGTGGCCCATGAGGCCCTGCCCAACAGGGTCACCGAGAGGACCGTGGACAAGTCCACCGGTAA
9521 ACCCACCCTGTACAACGTGTCCCTGGTCATGTCCGACACAGCTGGCACCTGCTACTGACCCTGCTGGCCT

9591 GCCCACAGGCTCGGGGCGGCTGGCCGCTCTGTGTGTGCATGCAAACTAACCGTGTCAACGGGGTGAGATG
9661 TTGCATCTTATAAAATTAGAAATAAAAAGATCCATTCAAAAGATACTGGTCCTGAGTGCACGATGCTCTG
9731 GCCTACTGGGGCGGCGGCTGTGCTGCACCCACCCTGCGCCTCCCCTGCAGAACACCTTCCTCCACAGCCC
9801 CCACCCCTGCCTCACCCACCTGCGTGCCTCAGTGGCTTCTAGAAACCCCTGAATTCCCTGCAGCTGCTCA
9871 CAGCAGGCTGACCTCAGACTTGCCATTCCTCCTACTGCTTCCAGAAAGAAAGCTGAAAGCAAGACCACAC
9941 GTATACAGGCAGCACACAGGCATGTGTGGATACACATGGACAGACACGGACACACACAAACACATGGACA
10011 CACAGAGACGTGCTAACCCATGGGCACACACATACACAGACATGGACCCACACACAAACATATGTGGACA
10081 CACATGTACAAACATGCACAGGCACACAAAGAGAACACTGACTACAGGCACACACACACACGGGCACACA
10151 CATGGATATGTGCACACATGGACACATACATGTGCAGGACATGCACACACACAGACACACTAGCACAGAG
10221 GCATACACACACAGACACACACATTCACAAACACACATGTGCATGCAAACACACACACATGTACAGACAC
10291 AAGTACATGGACACATGCACACCCAGAGACACACTGACACAGACACACAGGAGCATGTGATACACTAACA
10361 CGTGGACACACACGTCTACCCACAGGCACACAACAGATGGACACGCGTACACAGACATGCACACACCCAC
10431 AGGCACAACACGTGCGCATGCCGGCCGGCCCCCGCCCACATTCTCCCAGGGCCCTGCCGGATACTCTGTC
10501 CCTGCAGCAGTTTGCTCCCTGCGCTGTGCTGGCCCCGGGGCTTTGGGCCCAGGCTCTGCTTGTCCTTCTG
10571 TCTCTGCTTGGAGGTGCTGCCATGGCACCCAGCTTGGGCTCTGCCTGGGGAGCGGAGGCCCCAGGGATAG
10641 CATGTGACCCCTGCTGAGGCCAGGCTCCTGATGAAGGCAGCAGATAGCCCCCACACCCACCGGTGAGCAG
10711 AACCAGAGCCTGTGCCATGTGCTGAGAGCAGGCAGTGACTAAGCATATGGCCCAGAGGGCAGAGTGGCTG
10781 CCCTGGGCAGCTGCTCCTCTTAGCAGGAGGCCTCAGGAGATGAGCTAGAGCAAGTCTGCCCCTGCAAATA
10851 CCACCTGCTCCCCAACCCACAGCAGGGAGCAGGCGAGGTCAGACAGCAGCAGCCCGGGAAGGACCGAGCC
10921 CCAGCAGGGAAGGCAGGGCCCGAGTGAGGTCTCCACACCCAACGCACAGTGCTGTCTCTAACTGGGGCCA
10991 CCTCCGAGTCCCCGCCACACTCTTGGCCCTTTGGAGTCCTGGGCTCCAGGTGTCTCCCAAGGGCCCATCT
11061 GTGCAGGGGATGCAACCCCCCGAATGTCCTCATCCCACTGTGGAGCTCAGGTCTCTGTCTGCTCCCTGGG
11131 TCCTGGCAGGGTAGGACAAGTCCGCCAGGATGTCCCCATGCAGACTCTGCTCCAAGAGGGAGCTGGAGAG
11201 TCAGGGCCTTGGTGAGGGAGTCAGGATCGGGTTCCCCCCAGCTCAGTCCTCCCACCTGCCAGCCCCCACA
11271 GCACAGGGCAGGGCCACACCCCCTGCTTCCCCCTCCAGGAGAGTCAGGACATGCTGGCCGCTGCTCCGCT
11341 GGGGCCCCGCCCTCCAGCCCCCACCTTGGTCTGTGTGCTGCATCCCCCACGCTCTCTCTGCCACCCCAGG
11411 ACTCTGAGGAAAAGACCTCAGAGTCCCAGCCCTGCCCAGCCTCGGCCTGTGCCCCCGCTGCATCAGGCTT
11481 TCAGGGGCCCAGCCCATGCCCTGGGCAGTGCCCGAGCCCCCCTGCACTTGCTCTCCCCACCCCTGGGTGC
11551 AGCACAGCCTAGGGGCCAAGGGTGGGCCTA

Appendix 5. Location of switch recombination events in the mu locus. The position of J_H5, J_H6, the immunoglobulin enhancer (Eμ), and the mu constant region exons (Cμ1-4) are shaded in grey. The positions of switch recombination events are marked with the published clone name. The repeating pentamers GGGCT (blue) and GAGCT (magenta) are highlighted.

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   1 GGATCCCCGGCTGCAGGACAGTGACCTGGGAGTGAGTACAAGGTGAGGCCACCACTCAGGGTGCCAGCTC
  71 CAAGCGGGTCACAGGGACGAGGGCTGCGGCCATCAGGAGGCCCTGCACACACATCTGGGACACGCGCCCC
 141 GAGGGCCAGTTCACCTCAGTGCGCCTCATTCTCCTGCACAAAAGCGCCCCCATCCTTTCTTCACAAGGCT
 211 TTCGTGGAAGCAGAGGCGTCGATGCCCAGTACCCTCTCCCTTTCCCAGGCAACGGGACCCCAAGTTTGCT
 281 GACTGGGACCACCAAGCCACGCATGCGTCAAGAGTAGAGTCCGGGACCTAGGCAGGGGCCCTGGCGTTGG
 351 GCCTGAGAGAGAAGAGAACCTCCCCAGCACTCGGTGTGCATCGGTAGTGAAGGAGCCTCACCTGACCCCC
 421 GCTGTTGCTCAATCGACTTCCCAAGAACAGAGAGAAAAGGGAACTTCCAGGGCGGCCCGGGCCTCCTGGG
 491 GTTCCCACCCCATTTTTAGCTGAAAGCACTGAGGCAGAGCTCCCCCTACCCAGGCTCCACTGCCCGGCAC
 561 AGAAATAACAACCACGGTTACTGATCATCTGGGAGCTGTCCAGGAACCCGACAGAGCCGGAGGGCCACAC
 631 ATCCACAGGCACCAAATGGACGACCCGGCGCTTCAGGTATCCCAGCCCACCGGAGCCCCAATCGAGGGGC
 701 CGGGAGGCAGCTGTGAGGGAGAGGCTGCCTGGGCCCTGACAGCGAGGGACTCCTGGGCCACCTCACAGCA
 771 TCAACCAAGCTTCTTGGTCACTGCGTGGACTCTAGGCACTCCCTTCCCTGCATGGGGACACAGGAAAGCT
 841 AGAAACAGCCTAATGCCGACCTGGAGAAGGCAGGGGGCTGGGAATCTTGCTGGCCAAGACCAAGAAGTCA
 911 GAGACCCGACTGGCAGGGCTGGCTGGTGCAGACTGCCTGCCACCCCTAGGACAGGACAGCCAACCCAGAG
 981 CTGGAAGGGAGGGTGGGGAGGCGGCAGCAGGGAGCTGTCCTGAGCTCCACTGCGCAACTGGCTGATCTTG
1051 GCAAGTCCGAGCTGGGCGACTGAGGGGGGCTTGGCTGAGTGGACTAGACTGAGACGGGCCTAACAGACTG
1121 AGCTGAGGCGAGCTGGGTGGGCTGAGAGGGCTACCTGTCCCTTAGAGGACAGGTGGCCAAGCTGGGCTGT
                                                    pSC1.5-1(Sε/Sγ4)/pSC1.1-7(Sμ/Sε)
1191 CCTGAGCCAGGGCGATCGGGGCTGGCCCGGGCCAGGCGGGTTTAGCTGAGTTGAGTGAGTGGACTGGGTA
  pSC1.1-4(Sμ/Sε)                    pSC1.5-8(Sε/Sγ4)     pSC1.1-32(Sγ4/Sα1/Sε)
1261 GAGGGAAATGAGCTAGGCTCAGCTGAGCTAGGCTTGAGCTGGGTTATCCTAAGCCCTAAGGTGGACTAGC
pSC1.1-12(Sμ/Sε)
1331 TGGGCTGAGCTGGACTTATCTGGGCAGGCAAAGTCAGGCTGAGCTGAGGTGGCCTCCCTGGTGGTCCAGA
1401 TTGAGTTAAGCTGAATTAGGCTGACCTGGACTTGGTTGAAATAAGCTGGGCCGACACAGGAGTAGGGACA
                     pSC1.2-65/pSC1.1-35/pSC1.1-40(Sε/Sγ4)
1471 ACTACAGTTCTCTACTTAGGATAAAATGGGTGCTCGTGGACTATCCGGGCTGAAGGAGACCAAGCTGGGG
                     HJS7(Sμ/Sε)
                                                    2C10(Sμ/Sγ4/Sε) pSC1.6-5(Sε/Sγ4)
1611 AGCTTAGCTGGTTGGGCTGAGTAACTGGGCTGAGCTAAATGGGATTGAGCTGAGGAGGGCTAGGCTGGGG
                                                          pSC1.7-4(Sμ/Sε)
1681 GAGAGACTGACGACGGACAGGGTTAAAAGCTGGAGTGAGCAGGCCTTAAATTGGGCTGGGGTGATCTGAA
pSC1.7-6/pSC1.8-23(Sμ/Sε) pSC1.7-56(Sμ/Sγ4/Sε) pSC1.7-2(Sμ/Sε)
                                                pSC1.7-22(Sε/Sγ4) pSC1.8-10(Sμ/Sε)
1751 TTTAGCTGGGATGAGCTGGGCTGGGCTGAACTGTGCCCACGTGAACTGGGCTAAACTAGGCTCGCTGAGT
1821 GGACTCAGCTGGGTTGGTCTCTCGGGTTCAGCTGGGCTGGGCTGAGCTAGGTTAGACTGGGAAGCTGGGC
        pSC1.7-12(Sμ/Sε)
1891 TGGGTTGGGCTGGGTTGGGCTGGGCTGCTCTAGGCTAAGCTAACCTAACCTAACCTGGGTTCAGCTGGCC
SKS287(Sμ/Sε) pSC1.7-1(Sμ/Sε)
1961 TAGGTTGGGCTGGGTTGGGCTAAAGTCCCTTCAGCTGAGATATGCTAATATGGGCTGGGCTGGGTCAGGT
2031 TGAGGTTAACTGAACTGGGCTGACCTGGGCTGAGCTCAACTGAGTTCACATGGGCTGGGCTGGCCTGGCC
                                                          SKS288(Sμ/Sε)
2101 TGGGCCTAAACTGGGTTTGGCTGGGCTGGGCCAACTGGACTGAGGTGGATGGAGCTGGGCTGAGCTGGCC
2171 TGGCCGGGCCTGAGCTGTGATTGGAAGACCTGGGCTGAGCTGGACAGACCTGAGCCAAGCTTAGCTAGTT
                                SKS282(Sμ/Sε) SKS285(Sμ/Sε)
2241 GGGCTGACTAAGCTGGTTTGGGCTAAACTGGGTGAGCTGGGGAGGACTAGGCTGGGTGAGTGACCTGAGA
2311 TGGACAGGGTTATAAGAAGCTGGACTGAGCTGGGCTTGGATTATTGAACCGAATTGGGTTGGGGTGATTT
                                2C4/SKS281(Sμ/Sε)                SKS283(Sμ/Sε)
2381 AAACTGAGTTCTGCTGGGATAAGCTGATCTACTCTGGGCTGAACTGAGCAGAGCTGAACCTAGCTGGGCT
2451 GGGCTAAACTGGGCTAGCCTGAGCTGGGCTGAACTGGGCTGCTGGGCTGGACTGGGTAAGCTGGGCTGAG
                HJS6(Sμ/Sε)
2521 CTGGGTTGGGTGGAAATGGGCTGAGCTGAGCTAGGCTAAACTGGGTTTGGCTGGGCTGGGCTGGGCTGGG
2591 CTGGGTTCAGCTGAGCGGGTTGGGTTAGACTGGGTCAAACTGGTTCAGCTGAGATGGGCTGATATGAGCT
2661 GGGCTGGGCCAGGCTGAGCTCAGCTAAACTGGGCAGTGCTGGACTGGGCTGAGCTAGACTGGGCAACTGT
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                A5-1(Sμ/Sε)                                                                A5-2(Sμ/Sε)
                     |                                                                          |
2731 ATTCAGCTGGGCTGGCCTGGCCTCGGCTGGGCTGGGTTCAGCTGGGCTGGGCTGAGCAGGCCTGAGCAGG
2801 ATTAGTTGAGCTGGTCGTAACTGGATTTAACTAGCTAGGCTAGGCTTAACTGACTAAGCTGATCTGGACT
2871 GTATTCATCTGGATGAAGCTGGGGTGAGGTGGCTACTTTAGGTCAGCTTTGCTGAGCTAAACTGGACCGG
                                                                                SKS286(Sμ/Sε)
                                                                                     |
2941 GCTAAATTGATCTGGACTGACCATTCTCACCTGGCTAAGAGGAGCTGAGTCAGAAGCAAGCTGGTTGAGC
3011 TGGCTGGACTGAAATAAGAGTTTGCTGCCTGCAAGGGGAGGTCCTGGGCTGACCTGGGCCAGGCTGAACC
3081 AGGCTGGCTTAGAGTGAACTTCAGAGGGCGACTCCCCCGGTAGGCCAGTCTCAGCTGAACTTGGCTGTCC
                          U266(Sμ/Sε)
                               |
3151 CGGTGGGCAGAGCGGGGCTGGATACTGTGATTTTGGGGGTACCTAGAGCAGACTTCAAGACCAAGCTAAA
3221 CTGGGCTCCAGGGGCAGGATGGGCTGGGGACTTGGGACTCCAGGCCAGGGGCGAAGGGCCACGCTGTACA
3291 GACCGCACTATCTGGGCCAGGGTTCTGTGGTGGGAGGGACTGACTGCCTGGGGCATCAGGGCAAGTCTTC
3361 CCGCCCTCCCCTAGAGGTCAGGGGTGGGCAGAGCACCATGGGGGTCTGGCAGGTCAGGTGAGGGCTGCTG
3431 TGATGGGGAGATCCAGGCTTGGCACTCAAGAGCCCGAGGAGCTGAGACCACAGCCTTGGGGGGTTGGGGT
3501 CAGGGTTGGAGGGCAGGCAGACCATCCACCATGAGCCCAGAGAGAGTTTGAAGGGGGAGGGCTCTGGGGT
3571 CCGGCCCATGGGGTCCCTGGGTTTCAGCCTAGGGGCATGGCCCAGTGTCTCTGCTCCTGAGTGCCCACCG
3641 TGCAGCACTTGCAGGGGGAGGCTGGGGTCATCCTGGAGGCACCCCCCTTCCTGAGCCCAGCCTGATGATA
3711 GTGGCTGAGCAACAGCTTCTGGTGGGGGAATGGGCCCTGGGAGCCGCCCTGGGCCTGGGGATTGTGGGGA
3781 AAAAGGCCCAGAATGAGCCTAGCCATCTGGATCCCTGCCACGGGGTCCCCAGCTCCCCCATCCAGGCCCC
3851 CCAGGCCTGATGGGCGCTGGCCTGAGGCTGGCACTGACTAGGTTCTGTCCTCACAGCCTCCACACAGAGC
3921 CCATCCGTCTTCCCCTTGACCCGCTGCTGCAAAAACATTCCCTCCAATGCCACCTCCGTGACTCTGGGCT
3991 GCCTGGCCACGGGCTACTTCCCGGAGCCGGTGATGGTGACCTGCGACACAGGCTCCCTCAACGGGACAAC
4061 TATGACCTTACCAGCCACCACCCTCACGCTCTCTGGTCACTATGCCACCATCAGCTTGCTGACCGTCTCG
4131 GGTGCGTGGGCCAAGCAGATGTTCACCTGCCGTGTGGCACACACTCCATCGTCCACAGACTGGGTCGACA
4201 ACAAAACCTTCAGCGGTAAGAGAGGGCCAAGCTCAGAGACCACAGTTCCCAGGAGTGCCAGGCTGAGGGC
4271 TGGCAGAGTGGGCAGGGGTTGAGGGGGTGGGTGGGCTCAAACGTGGGAACACCCAGCATGCCTGGGGACC
4341 CGGGCCAGGACGTGGGGGCAAGAGGAGGGCACACAGAGCTCAGAGAGGCCAACAACCCTCATGACCACCA
4411 GCTCTCCCCCAGTCTGCTCCAGGGACTTCACCCCGCCCACCGTGAAGATCTTACAGTCGTCCTGCGACGG
4481 CGGCGGGCACTTCCCCCCGACCATCCAGCTCCTGTGCCTCGTCTCTGGGTACACCCCAGGGACTATCAAC
4551 ATCACCTGGCTGGAGGACGGGCAGGTCATGGACGTGGACTTGTCCACCGCCTCTACCACGCAGGAGGGTG
4621 AGCTGGCCTCCACACAAAGCGAGCTCACCCTCAGCCAGAAGCACTGGCTGTCAGACCGCACCTACACCTG
4691 CCAGGTCACCTATCAAGGTCACACCTTTGAGGACAGCACCAAGAAGTGTGCAGGTACGTTCCCACCTGCC
4761 CTGGTGGCCGCCACGGAGGCCAGAGAAGAGGGGCGGGTGGGCCTCACACAGCCCTCCGGTGTACCACAGA
4831 TTCCAACCCGAGAGGGGTGAGCGCCTACCTAAGCCGGCCCAGCCCGTTCGACCTGTTCATCCGCAAGTCG
4901 CCCACGATCACCTGTCTGGTGGTGGACCTGGCACCCAGCAAGGGGACCGTGAACCTGACCTGGTCCCGGG
4971 CCAGTGGGAAGCCTGTGAACCACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCACGTTAACCGTCAC
5041 GTCCACCCTGCCGGTGGGCACCCGAGACTGGATCGAGGGGGAGACCTACCAGTGCAGGGTGACCCACCCC
5111 CACCTGCCCAGGGCCCTCATGCGGTCCACGACCAAGACCAGCGGTGAGCCATGGGCAGGCCGGGGTCGTG
5181 GGGGAAGGGAGGGAGCGAGTGAGCGGGGCCCGGGCTGACCCCACGTCTGGCCACAGGCCCGCGTGCTGCC
5251 CCGGAAGTCTATGCGTTTGCGACGCCGGAGTGGCCGGGGAGCCGGGACAAGCGCACCCTCGCCTGCCTGA
5321 TCCAGAACTTCATGCCTGAGGACATCTCGGTGCAGTGGCTGCACAACGAGGTGCAGCTCCCGGACGCCCG
5391 GCACAGCACGACGCAGCCCCGCAAGACCAAGGGCTCCGGCTTCTTCGTCTTCAGCCGCCTGGAGGTGACC
5461 AGGGCCGAATGGGAGCAGAAAGATGAGTTCATCTGCCGTGCAGTCCATGAGGCAGCGAGCCCCTCACAGA
5531 CCGTCCAGCGAGCGGTGTCTGTAAATCCCGGTAAATGACGTACTCCTGCCTCCCTCCCTCCCAGGGCTCC
5601 ATCCAGCTGTGCAGTGGGGAGGACTGGCCAGACCTTCTGTCCACTGTTGCAATGACCCCAGGAAGCTACC
5671 CCCAATAAACTGTGCCTGCTCAGAGCCCCAGGTACACCCATTCTTGGGAGCGGGCAGGGCTGTGGGCAGG
5741 TGCATCTTGGCACAGAGGAATGGCCCCCAGGAGGGGCAGTGGGAGGAGGTGGGCAGGGCTGAGCCCCCCC
5811 TGGAGAGGCGGTGGGAGGAGGTGGGCAGGGCTGAGGTGCCACTCATCCATCTGCCTTCGTGTCAGGGTTA
5881 TTTGTCAAACAGCATATCTGCAGGGACTCATCACAGCTACCCCGGGCCCTCTCTGCCCCCACTCTGGGTC
5951 TACCCCCTCCAAGGAGTCCAAAGACCCAGGGGAGGTCCTCAGGGAAGGGGCAAGGGAGCCCACAGCCCTC
6021 TCTCTTGGGGGCTTGGCTTCTACCCCCCTGGACAGGAGCCCCTGCACCCCCAGGTATAGATGGGCA
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Appendix 6. Location of switch recombination events in the epsilon locus. The position of the germline-ε exon (GL-ε), and the epsilon constant region exons (Cε1-4) are shaded in grey. The positions of switch recombination events are marked with the published clone name. The repeating pentamers GGGCT (blue) and GAGCT (magenta) are highlighted.

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Our work provides strong evidence that local regulation of IgE synthesis by IL-4 may occur in nasal B cells. Further studies are needed to strengthen this conclusion, but there are several implications: local regulation of IgE synthesis could account for the efficacy of topical steroid treatment in hayfever patients, and could be the basis for the development of new therapeutic strategies. It may also explain why only a proportion of "atopic" individuals (with a genetic predisposition to exhibit exaggerated IgE responses) develop hayfever, while others have either no clinical manifestations or develop atopic disease (such as asthma or eczema) in other anatomical locations.

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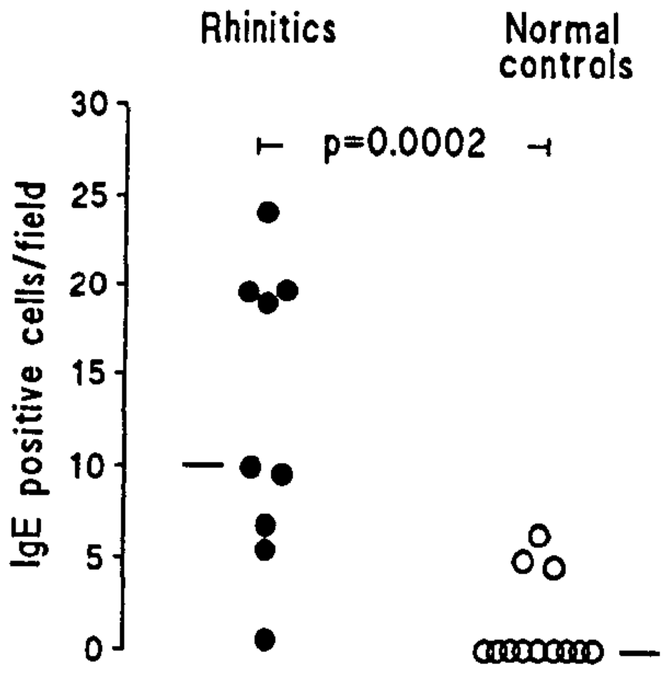

Figure 6. Results of immunohistology to detect IgE in nasal biopsy specimens. An anti-IgE Ab was used to detect IgE in nasal biopsy samples obtained outside the pollen season from grass pollen-sensitive hayfever patients (●) and non-atopic normal control subjects (○). Solid bars represent median values.

Our failure to detect IgE protein within nasal B cells by immunohistochemical methods may be a consequence of low levels of IgE expression and/or of secretion rather than intracellular accumulation of a major portion of the product. It may be that relatively few committed B cells go on to secrete IgE in large amounts. This would be consistent with our failure to detect Cε-positive $CD38^+$ plasma cells in the nasal biopsy sections. It is possible, on the other hand, that there are too few plasma cells secreting large amounts of IgE to have been detected in our assay samples. Very few plasma cells may, however, secrete large amounts of IgE. Bonnefoy and co-workers [38] were unable to detect IgE in sections from lung-associated lymphoid tissue, but found that IgE was produced by the isolated tissue in culture. The synthesis of mRNA by nasal B cells is *prima facie* evidence of protein synthesis.

The present study is only the second in which germ-line gene transcripts have been observed *in vivo*. Iα transcripts were previously observed by Cebra and co-workers [39] in Peyer's patches isolated from mouse gut. However, Peyer's patches are recognized as gut-associated lymphoid tissue, whereas the nasal mucosa is not a previously recognized locus for heavy chain switching. The expression of Iε transcripts by nasal B cells is consistent with the view that switching occurs in this tissue, but further proof is required.

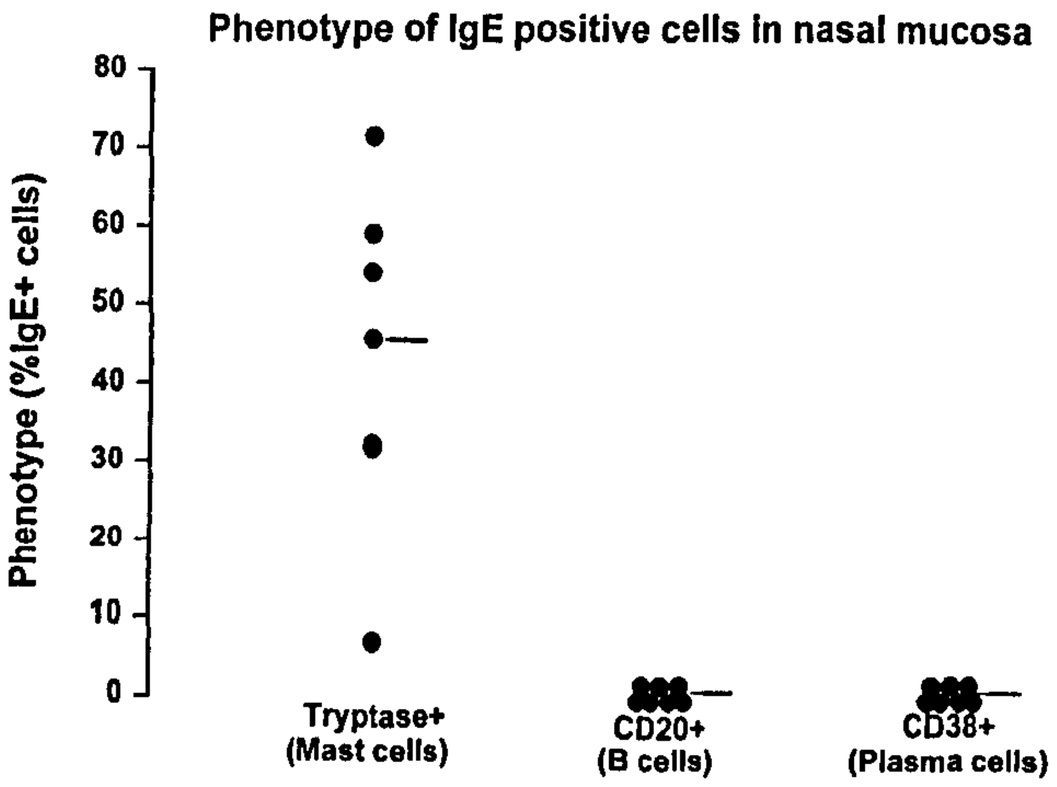

Figure 7. Colocalization of IgE with mast cell and B cell markers. Biopsies from the nasal mucosa of hayfever patients were probed by double immunohistochemistry, using anti-IgE and phenotypic markers for mast cells (anti-tryptase) B cells (anti-CD20) and plasma cells (anti-CD38).

Our results (Figs. 3–5) substantially reinforce the hitherto fragmentary evidence for local regulation of IgE synthesis. Since IL-4 is required for targeting class switching to IgE, it may be conjectured that the allergen-induced elevation of IL-4 gene expression in nasal T cells and/or mast cells is responsible for the Iε transcripts observed at the same time in neighboring B cells. The inhibition of IL-4 gene transcription by FP eliminates both the increase in $IL\text{-}4^+$ and $I\varepsilon^+$ cells in response to allergen, in accord with the conclusion that IL-4 induces Iε transcription. FP has been shown to have no systemic bioavailability [40], and must therefore exert its effects locally. FP therapy did not cause any obvious reduction in the background of $C\varepsilon^+$ cells (Fig. 5A), and may therefore be ineffective towards cells already committed to IgE synthesis. However, it could also alter the ratio of ε chain mRNA for the membrane-bound and secreted forms of IgE, by analogy with other known perturbations of B cells [3–5, 41]. Further work is required to investigate this possibility. Until this is done, we cannot speculate on the relationship between local IgE and the improvement in patients' symptoms.

The second signaling agent required for heavy chain switching to IgE in B cells is CD40L. Transient activation of CD40L expression is induced by antigen presentation to T cells. Allergen challenge may therefore have another critical consequence for local switching to IgE in uncommitted B cells if there is also a local source of IgE. Allergen can bind to IgE antibodies via IgE receptors on APC, leading to antigen presentation to T cells [42]. Our results (Figs. 4 and 7) demonstrate that IgE may be found at higher levels in the nasal mucosa of hayfever patients between seasons than in normal individuals. On allergen challenge or on arrival of the pollen season, this IgE may act to prime APC for presentation of allergen to Th2 cells, and so enable them to express IL-4 and CD40L simultaneously and form the necessary contact with local B cells to induce switching to IgE. Local IgE may influence the allergic status of an individual by its effects on APC as well as mast cells.

Local switching would not exclude the possibilities that some of the $C\varepsilon^+$ B cells are generated elsewhere and migrate to the tissue, or that mast cells and basophils transport IgE to the tissue. In a study of IgE trafficking in the rat, Bell and co-workers [43] observed that IL-4 induced the specific transport of circulating IgE to the gut. The relative contributions of the different mechanisms by which mast cells and APC in the nasal mucosa accumulate IgE cannot be resolved by the present methods: neither trafficking studies to detect cell populations homing to the nasal mucosa, nor kinetic studies, using serial biopsy samples from a single patient to obtain stronger circumstantial eidence for local switching, would gain ethical approval because they are too invasive. Future experiments are planned with nasal biopsy samples cultured *ex vivo*.

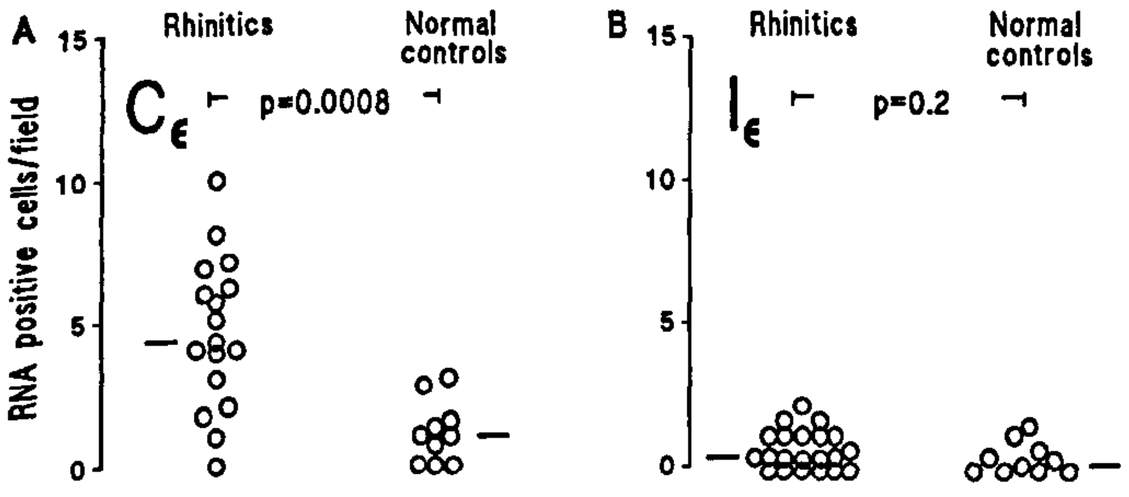

Figure 4. Comparison of ε gene transcripts in hayfever patients and non-atopic normal control subjects. Results of *in situ* hybridization using antisense probes for RNA for Cε (A) and Iε (B) of nasal biopsy samples outside the pollen season in hayfever patients and non-atopic normal control subjects are shown.

(tryptase positive), and there was no detectable localization to either $CD20^+$ B cells or $CD38^+$ plasma cells (Fig. 7).

4 Discussion

Following the recognition of IgE as the "reaginic antibody" [30], IgE was detected in nasal secretions [31–34]; this raised the possibility that there might be local IgE synthesis. Ohashi et al. further detected IgE in nasal tissue homogenates [35]. Platts Mills [34] demonstrated grass pollen-specific IgE in nasal but not salivary secretions of hayfever patients. Huggins and Brostoff [31] suspected local IgE synthesis on clinical grounds in some patients with clear-cut seasonal hayfever symptoms but no positive skin prick test or detectable grass pollen-specific IgE in the serum. Grass pollen sensitivity was confirmed by nasal provocation with a grass pollen extract, and specific IgE was detectable in nasal fluid in amounts comparable to those in hayfever sufferers, who also showed positive skin tests and elevated allergen-specific serum IgE. However, these observations, while demonstrating the presence of IgE, did not establish whether the IgE resulted from plasma exudation of IgE, from the recruitment of cells carrying IgE on surface receptors, from migratory IgE-producing plasma cells, or from *in situ* production of IgE by B cells in the nasal mucosa. The last of these possibilities was examined by Ganzer and Bachert [36] who, by use of immunohistology, were able to detect small numbers of IgE^+ B cells in cervical glands and tonsillar tissue, but not the nasal mucosa of hayfever patients.

Here we have shown that the ε gene is expressed in nasal B cells. In hayfever patients between seasons we found only transcripts of the rearranged ε gene (ε chain mRNA), but after local allergen provocation we observed germ-line gene transcripts in additional cells. The proportion of B cells that express ε chain mRNA appears to be extremely high (assuming that $CD20^+$ cell counts in Table 1 represent a large proportion of the total B cell population), certainly greater than the 1 in 10000 cells reported to be IgE^+ in the circulation [37]. The distorted isotype ratio is confirmed by our observation that comparable numbers of cells express Cε RNA and Cγ RNA (Fig. 4A, D). Interestingly, the population of $mRNA^+$ cells within the nasal mucosa in hayfever patients appears to be several times greater than that found in normal individuals: this may be diagnostic of their condition.

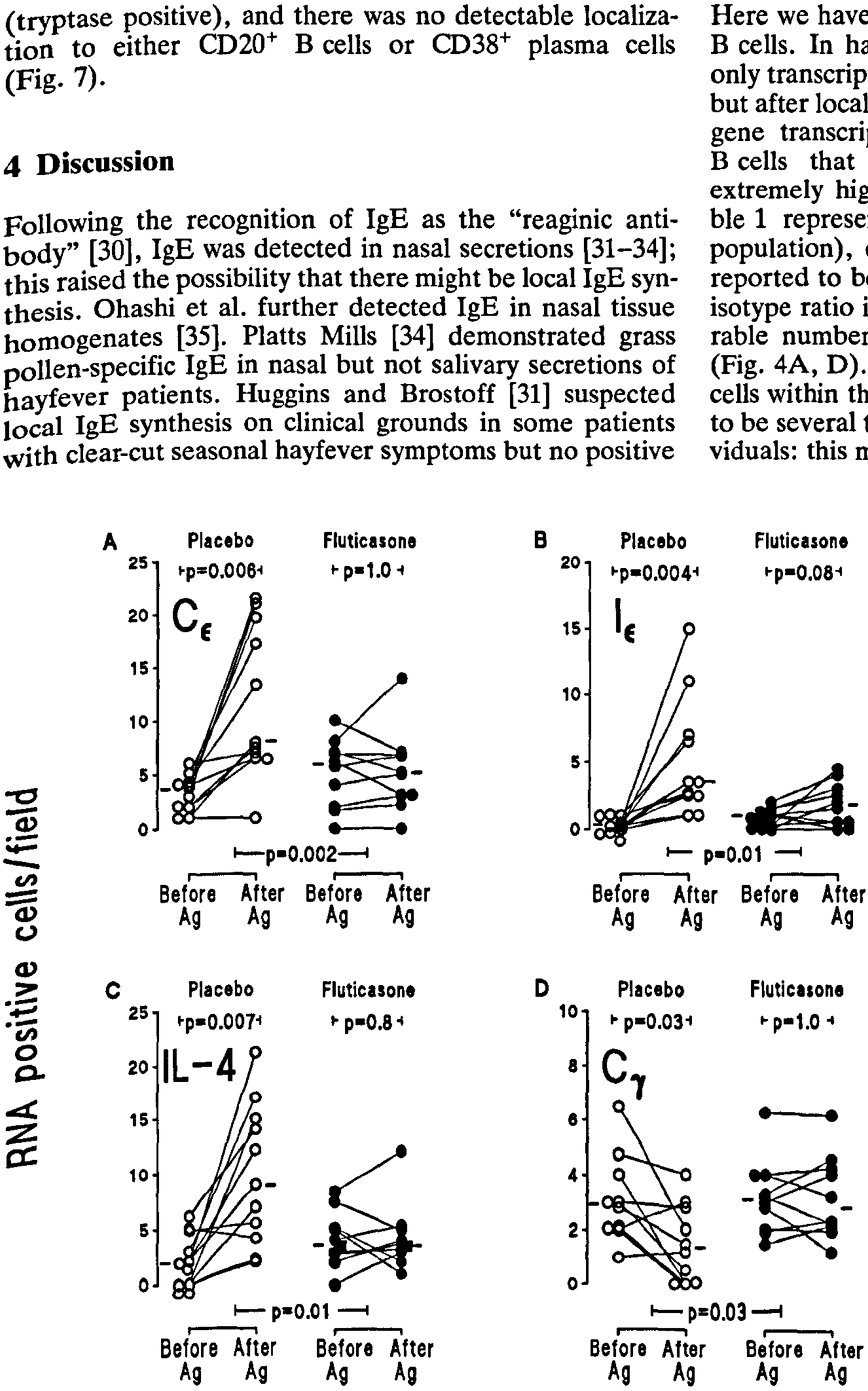

Figure 5. In situ hybridization using antisense probes for RNA in nasal biopsy samples obtained before and after nasal allergen provocation. (A, B) Cε and Iε in placebo- and topical corticosteroid-treated patients. Cγ and IL-4 $mRNA^+$ cells were also examined in the same samples (C, D). Solid horizontal bars represent median values. Within-group comparisons (before/after allergen) were made using the Wilcoxon test. Between-group comparisons (FP *vs.* placebo) were made using the Mann-Whitney U Test. Levels of statistical significance are shown.

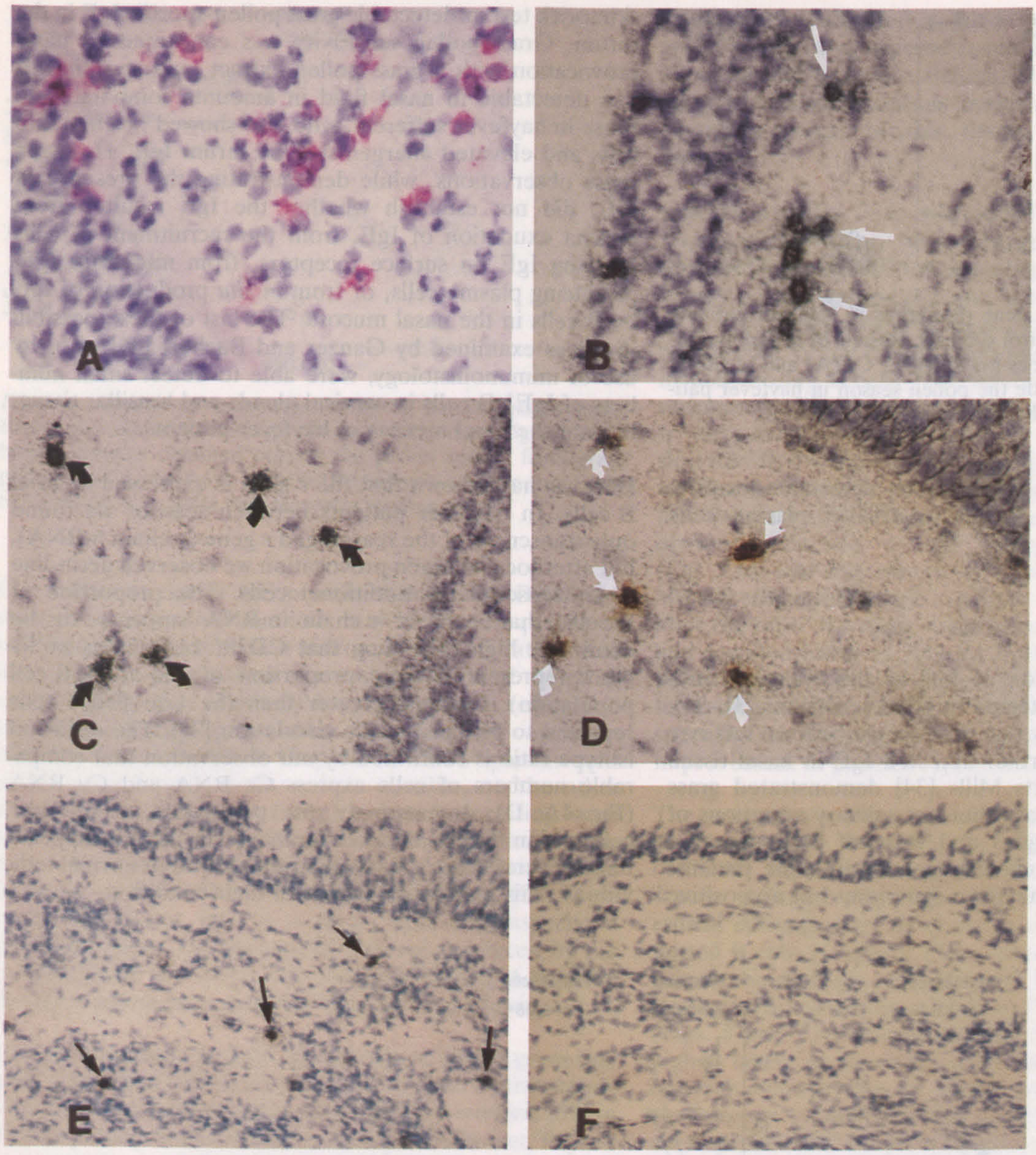

Figure 3. Immunohistology and *in situ* hybridization of nasal biopsy specimens. (A) $CD20^+$ B cells, (B) IL-4 $mRNA^+$ cells, (C) IgE $C\varepsilon^+$ cells after allergen challenge which are colocalized to $CD20^+$ cells, (D) by use of double immunohistochemistry/*in situ* hybridization. Iε expression after allergen is compared in placebo and topical corticosteroid-treated patients in panels (E) and (F).

Increases in the number of cells expressing Iε and Cε RNA were accompanied by a rise in the number expressing IL-4 RNA (Fig. 5C). However, the magnitudes of these effects were not correlated, reflecting the fact that IL-4 expression occurs in different cell types, whereas the parallel increases in numbers of $C\varepsilon^+$ and $I\varepsilon^+$ cells are likely to refer not only to the same cell type but to identical cells.

A Cγ probe was used to compare the effect of allergen challenge on expression of ε and γ chain genes in the hayfever patients. In contrast to the ratio of the immunoglobulins in serum (IgG:IgE = 10000:1), the populations of $C\gamma^+$ and $C\varepsilon^+$ cells in the nasal mucosa before allergen challenge were about equal. Furthermore, allergen challenge resulted in a significant *decrease* in the $C\gamma^+$ population, contrasting to an *increase* in $C\varepsilon^+$ cells (Fig. 5A, D).

3.5 Effects of topical corticosteroid therapy in hayfever patients

Topical corticosteroid therapy resulted in inhibition of these allergen-induced changes within the nasal mucosa (Fig. 5A–D). Thus, in FP-treated patients there were no significant changes in either Cε, Iε, IL-4 or Cγ RNA^+ cells. The differences between them and placebo-treated patients were all highly significant (Fig. 5).

3.6 Immunolocalization of IgE

IgE protein-positive cells were more numerous in biopsy samples from atopic, grass pollen-sensitive patients outside the pollen season when compared with non-atopic normal subjects (Fig. 6). Double immunihistochemistry revealed that the majority of these cells were mast cells

rank method. Statistics were performed using a statistical package (Minitab Statistical Software, Minitab, In. PA 16801-2756, USA). p values less than 0.05 were taken as significant.

3 Results

3.1 Clinical data

The clinical features of the 21 patients with summer hayfever examined in this study are shown in Table 1. Patients treated with placebo or topical corticosteroid (FP) were well matched for age, gender and circulating grass pollen-specific IgE concentration. Clinical results of nasal allergen provocation are summarized in Fig. 2. Allergen provocation resulted in early and late increases in sneezing, nasal blockage and secretions (all $p < 0.01$) in the placebo-treated group. These effects were all markedly reduced in the topical steroid-treated group.

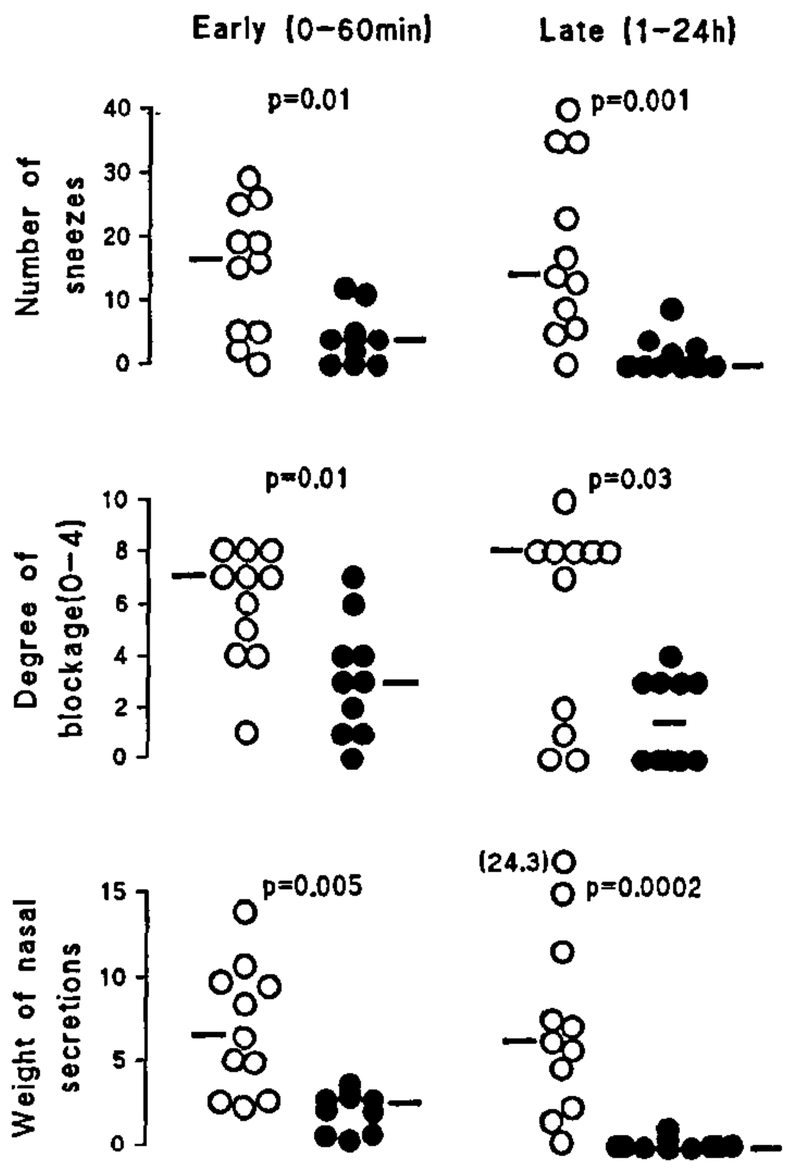

Figure 2. Nasal allergen provocation tests. Data are presented as individual values after FP (●) or placebo (○) nasal spray. All symptoms (early and late) were inhibited by topical corticosteroid (all $p < 0.01$, except late blockage $p < 0.03$; Mann-Whitney U Test).

3.2 Colocalization of ε gene transcripts to B cells

$CD20^+$ B cells were present in the nasal epithelium and submucosa in about the same numbers in atopic and normal subjects and did not change after allergen challenge (Table 1, Fig. 3A). Each $C\varepsilon^+$ cell was identified by a discrete cluster of silver grains (Fig. 3C). In three individuals, double immunostaining/*in situ* hybridization showed that more than 95% of $C\varepsilon^+$ cells were identified as $CD20^+$ B cells (Fig. 3D). From typical sections such as this it was apparent that a high proportion of the B cells in this tissue express the ε chain gene.

3.3 Expression of Cε and Iε RNA in hayfever patients and normal controls

The numbers of $C\varepsilon^+$ and $I\varepsilon^+$ cells in the nasal mucosa were examined in hayfever patients out of season and compared with corresponding cell counts in normal healthy control subjects. No cells positive for Iε were detected in either group. Interestingly, however, the mean value for the number of $C\varepsilon^+$ ($mRNA^+$) cells was several times higher in hayfever patients than in normal controls (Fig. 4).

3.4 Effect of local allergen challenge on expression of Iε, Cε, Cγ and IL-4 RNA in hayfever patients

Patients with hayfever showed a marked increase in the number of $C\varepsilon^+$ cells 24 h after allergen challenge (Fig. 5A) and $I\varepsilon^+$ cells became clearly detectable (Fig. 5B). There was a significant positive correlation between the number of $I\varepsilon^+$ cells and the increase in $C\varepsilon^+$ cells ($r = 0.76$, $p < 0.01$); *i.e.* the number of $I\varepsilon^+$ cells was similar to the increase in the number of $C\varepsilon^+$ cells in individual patients. Simultaneous *in situ* hybridization of allergen-challenged biopsy samples from four patients with Cε and Iε probes revealed that all $I\varepsilon^+$ cells were also $C\varepsilon^+$. However, only 65% (60–70%) of $C\varepsilon^+$ cells were $C\varepsilon^+$ and $I\varepsilon^+$, *i.e.* a mean of 35% of $C\varepsilon^+$ cells were $C\varepsilon^+$ and $I\varepsilon^-$. These findings demonstrate that the two probes are equally sensitive and that $I\varepsilon^- C\varepsilon^+$ RNA is ε chain mRNA (Sect. 1.2).

Table 1. Clinical features of 21 patients with summer hayfever

		Placebo	FP
Number		11	10
Age (years)		31.3 (2.7)	30.0 (2.4)
Gender		6M, 5F	4M, 6F
Serum total IgE (KU/l)		50.9 (17)	92 (23)
Serum grass pollen-specific IgE (Phadebas scale 0–5)		2.7 (0.4)	3.0 (0.3)
B cells ($CD20^+$) in nasal Mucosa (cells/hpf)[a)]	Before allergen	7 (3–17)	7 (1–21)
	After allergen	2 (1–30)	2 (1–12)

a) B cell counts are expressed as medians (interquartile ranges) per high power field (hpf).

with antisense riboprobes, following pretreatment of sections with RNase as described [19]. Controls were performed with sense probes (having an identical sequence to messenger RNA) (Fig. 3B, C, E, F).

Immunohistochemical staining for IgE was performed on biopsy specimens from 9 grass pollen-sensitive patients and 12 non-atopic normal subjects outside the pollen season. Double immunohistochemistry was performed on biopsy specimens from 7 pollen-sensitive patients using anti-IgE (immunoperoxidase method, in which positive cells stain brown), followed by the phenotypic Ab markers (APAAP method, in which positive cells stain red), resulting in double-positive cells, which stain red-brown [25, 26].

Tissue sections were assessed using an Olympus BH2 microscope with an eyepiece graticule at 200 × magnification. Counts were made along the whole length of nasal biopsy samples to a depth of 0.45 mm (including the whole samples lamina propria, where the majority of inflammatory cells were located). Counts were expressed per high-power field (hpf, 0.202 mm^2). The coefficient of variation between observers was <5 % for *in situ* hybridization and 8 % for immunohistology counts.

2.3 Genetic probes

Our Iε probe (Fig. 1) corresponded to the sequence from the start site of ε germ-line gene transcription, referenced as nucleotide 1, to the end of the Iε exon at position 650. This was generated by cloning the indicated Bam HI fragment (top line; accession no. X56797) into Bluescript Ksp. The plasmid, linearized with Xba I or Hind III, was transcribed in the presence of [^{32}P]UTP with T7 polymerase for the antisense probe and with T2 polymerase for the sense probe used as a negative control [27].

The Cε probe was generated from an ε chain cDNA clone [28]. The sequence corresponded to a 1150-base Hind III fragment from pSC213, encompassing 24 bases of Cε1 and the rest of the Cε sequence. This fragment was cloned into pGEM [23], cut at a Sma I site and transcribed with SP6 polymerase for the antisense probe (526 nucleotides), and cut at the Bam HI site and transcribed with T7 polymerase for the sense probe, used as a negative control.

For the consensus γ chain constant region probe (Cγ), a 5′ end-labeled 30-base oligonucleotide was used, corresponding to the Cγ2 region (IgG1, nucleotide 1206–1235 of accession no. J00228; IgG2, nucleotide 1204–1233 of J00230; IgG3, nucleotide 1785–1814 of X03604; IgG4, nucleotide 1205–1234 of K01316). The consensus Cγ probe had complete homology to all four IgG subclasses.

The IL-4 probe was prepared by inserting a human IL-4 cDNA of 500 bp (generously provided by Dr. J.-Y. Bonnefoy, Glaxo Institute for Molecular Biology, Geneva, Switzerland) into pGEM as described above.

2.4 *In situ* hybridization

Double immunostaining and *in situ* hybridization [20] (see Fig. 3D) were performed with anti-CD20 Ab, visualized by diaminobenzidine (DAB) brown staining. *In situ* hybridization was then performed using a ^{35}S-labeled complementary RNA probe, followed by autoradiography. Individual double-positive cells were evident as brown cells within a clump of black silver grains at 400 × magnification.

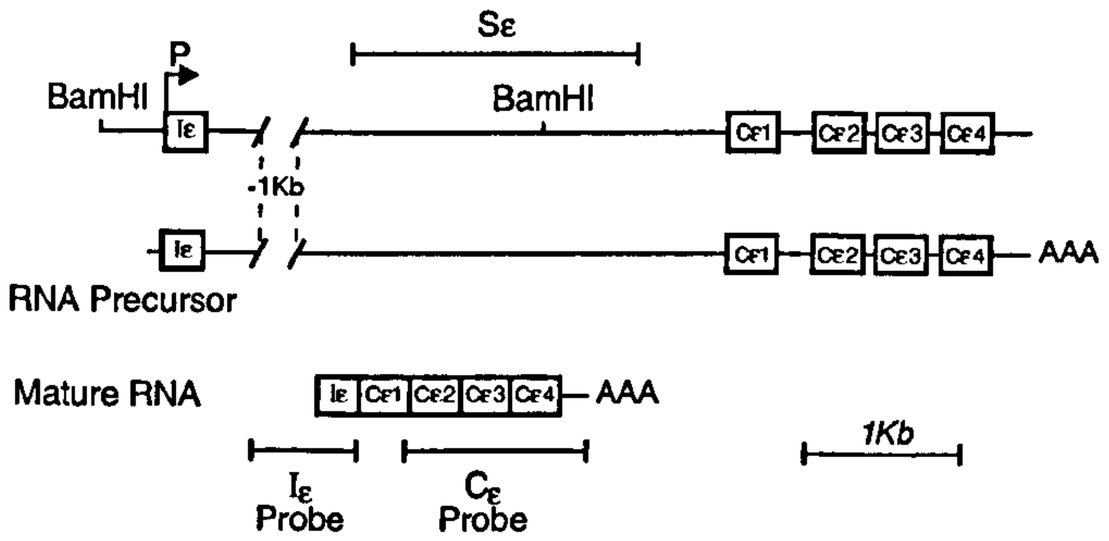

Figure 1. Identity of the Iε and Cε and probes. Top line: the ε germ-line locus (DNA), showing sites of digestion with Bam HI, the start site of transcription (P), the switch region (Sε) and the Iε exon and the exons encoding the four ε chain constant regions (Cε1–4). Middle line: the unprocessed ε germ-line gene transcript. Bottom line: the mature RNA ε germ-line gene transcript after RNA splicing, and the sequences corresponding to the Iε and Cε probes. Exposure to IL-4 induces transcription of the ε germ-line gene from the promoter site (P). Transcription proceeds through the ε chain constant region-coding exons Cε1–Cε4, but RNA splicing joins the Iε and Cε1 exons. When genetic recombination occurs, the DNA between the recombined sequences in the homologous switch regions (Sε), including the Iε exon, are deleted and replaced by a transcription unit containing the ε chain variable region coding exons. The Iε probe thus detects the transient appearance of ε germ-line gene transcripts, which precedes rearrangement of the gene and the synthesis of mRNA. The Cε probe detects both the ε germ-line transcript and ε chain mRNA.

Simultaneous *in situ* hybridization with probes coding for Cε and Iε was performed as described [29]. In brief, the Cε probe was labeled with digoxigenin and the Iε probe with ^{35}S-labeled UTP. The probes were applied simultaneously to 10-μm cryostat sections of nasal biopsy samples from four patients who had received placebo nasal treatment at 24 h after allergen provocation with grass pollen extract. Hybridization was performed at 42 °C for 12 h. The Cε probe was visualized with antibody to digoxigenin labeled with chromogen. Slides were then exposed for 10 days and Iε RNA was visualized by autoradiography.

2.5 Statistical analysis

Patients were stratified according to their level of skin prick test sensitivity to grass and hayfever severity before random selection to receive either FP or matched placebo nasal spray. This resulted in closely matched groups for comparison (Table 1). All biopsy samples were coded in random order and sections counted blind, independently of the clinical protocol. Within-group comparisons (baseline/allergen) were based on the Wilcoxon matched-pairs signed-ranks test. Between-group comparisons (magnitude of changes between baseline and allergen for subjects on FP compared with those on placebo nasal spray) were performed using an unpaired analysis (Mann-Whitney U test). Correlations were performed using Spearman's

at 24 h. We and others [22] have also found that nasal tissue harbors a population of B cells, the extent of which is unaffected by allergen challenge. In a double-blind trial we further showed that pretreatment with the topical corticosteroid, fluticasone propionate (FP), inhibits allergen-induced early and late nasal responses, as well as the associated recruitment of Th2 cells and expression of IL-4 [23]. This suggested that the release of IL-4 by mast cells and/or T cells on activation by allergens may induce heavy chain switching to IgE *in situ* and that some of the committed cells may go on to secrete IgE.

To resolve this question in respect of nasal tissue, we have re-examined the number of B cells and IL-4-expressing cells in the nasal mucosa of hayfever patients exposed to allergen, including those pretreated with FP, using previously described methods of immunohistology and *in situ* hybridization on nasal biopsy sections [19, 20, 24]. In addition we have used new RNA probes to detect whether resident B cells have the capacity to express IgE or undergo allergen-induced local switch recombination to IgE.

1.2 Experimental approach

IL-4 and IL-13 direct heavy chain switching to IgE by stimulating the transcription of the ε germ-line gene [8]. Germline gene transcripts contain a specific sequence, the Iε sequence, which is spliced to the sequence that codes for the constant region of the ε chain (Cε1–Cε4) (Fig. 1). However, the Iε exon contains a number of stop codons and hence the germ-line gene transcript is not translated into protein. The Iε exon is deleted in the switch, so that the Iε sequence is absent from ε chain mRNA transcribed from the rearranged gene. Thus it provides a specific probe for the detection of the ε germ-line gene transcripts. We have been able to determine in this way whether the increase in IL-4 mRNA (and presumably protein), induced by allergen challenge in the nasal mucosa of hayfever patients, indeed leads to the appearance of ε germ-line gene transcripts in neighboring B cells, as we predicted.

Probing for transcripts of the rearranged gene presents greater difficulty. We have used an ε chain constant region probe, the Cε probe, complementary to the sequence in ε chain mRNA that codes for the Cε2–Cε4 region of the ε chain. However, this sequence also occurs in germ-line gene transcripts. These contain a single copy of Iε and Cε2–Cε4 sequences (Fig. 1), and the two probes which we have prepared are of similar lengths (650 and 526 nucleotides, respectively; Sect. 2.3). When labeled under the same conditions they should therefore afford comparable sensitivity. Sensitivity may not be an issue anyway, since we measured the number of positive cells, not the amounts of RNA per cell. All cells that contained RNA in excess of the lower limit of detection were counted. The difference between the numbers of cells detected with the Cε and Iε probes was then the number that express ε chain mRNA. Several experimental observations justified the subtraction procedure. Thus, double *in situ* hybridization of nasal biopsy sections with the Iε and Cε probes revealed that essentially all $I\varepsilon^+$ cells are also $C\varepsilon^+$, but not *vice versa* (Sect. 3.4). Moreover, we found that nasal biopsy samples from hayfever patients between seasons and normal individuals contained only $C\varepsilon^+$ cells, but after allergen challenge there was an *increase* in the number of $C\varepsilon^+$ cells above this background, equal to the total number of $I\varepsilon^+$ cells (Sect. 3.4 below).

We have used the combination of Iε, Cε and IL-4 probes to determine the concentrations of B cells expressing ε germline gene transcripts, ε chain mRNA and IL-4 mRNA in the nasal mucosa of hayfever patients before and 24 h after allergen challenge, and after 6 weeks treatment with FP. Parallel experiments were carried out with a probe for γ chains (the Cγ probe) to compare the effects of allergen challenge and steroid treatment on expression of ε and γ chain genes. We have also used double immunohistochemical staining with differentially labeled antibodies against IgE plus either CD20 (for B cells), CD38 (for plasma cells) or tryptase (for mast cells) in an attempt to distinguish IgE associated with these cell types in the nasal biopsy sections.

2 Materials and methods

2.1 Patients

The study was performed with the approval of the Royal Brompton Hospital Ethics Committee and the patients' written informed consent. Twenty-one patients with summer hayfever were studied. Inclusion criteria for the patients included a history of at least 2 years of summer hayfever and a positive skin prick test (greater than 5 mm) to Timothy grass pollen extract (*Phleum pratense,* Soluprick, ALK, Denmark). Patients were all non-smokers. None had received topical or oral medication in the previous 6 months, or immunotherapy in the previous 5 years. Normal subjects were non-atopic (negative skin test) non-smokers with no nasal symptoms. Baseline blood samples and nasal biopsies were performed out of season at a time when the patients were asymptomatic. Patients were randomized to receive 6-week pretreatment with topical nasal corticosteroid (200 mg FP, two sprays per day) or a matched placebo nasal spray containing the aqueous diluent. After treatment all patients underwent local nasal provocation with grass pollen extract, followed by a second nasal biopsy after 24 h. Nasal provocation was performed with 1000 biological U of grass pollen extract (*P. pratense,* ALK, Denmark) applied on a 4-mm filter paper disc to the under-surface of the inferior nasal turbinate. Nasal biopsies were taken from the site of allergen challenge using 2.5-mm diameter cup-and-ring forceps (Gerritsma forceps) as described [19].

2.2 Immunohistology

The mAb used were anti-CD20 for B cells (Dako Ltd., High Wycombe, GB), anti-IgE (Vectra Ltd., GB), anti-tryptase antibody G3 for mast cells (kindly provided by Dr. L. Schwartz, Richmond, VA), and anti-CD38 antibody for plasma cells (Dako).

Immunohistology of nasal biopsies was performed on 6-μm cryostat sections using a modified alkaline phosphatase anti-alkaline phosphatase (APAAP) method [24] (Fig. 3A). *In situ* hybridization was performed on 10-μm cryostat sections, pre-fixed in 4% paraformaldehyde and hybridized

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Expression of ε germ-line gene transcripts and mRNA for the ε heavy chain of IgE in nasal B cells and the effects of topical corticosteroid

We have studied the expression of the gene encoding the ε heavy chain of IgE in nasal B cells of hayfever patients. We developed probes to detect transcripts of the ε germ-line gene and the rearranged gene by *in situ* hybridization of biopsy sections from the nasal mucosa. We compared tissue from hayfever patients out of season with that of normal controls, and also of hayfever patients treated with topical corticosteroid (fluticasone propionate) or placebo for 6 weeks and then challenged with antigen. ε chain mRNA was expressed in an unexpectedly high proportion of nasal B cells of both hayfever patients and normal subjects. However, although similar numbers of B cells were found in both groups, the proportion of cells that express ε chain mRNA was several times higher in the hayfever patients. No transcripts of the ε germ-line gene were detected in either group before allergen challenge. When hayfever patients were administered antigen locally, early (10–30 min) and late (1–24 h) symptoms ensued. After 24 h, coincident with an increase in the number of cells expressing mRNA for IL-4 in the tissue, ε germ-line gene transcripts appeared in the nasal B cells. The induction by allergen of IL-4 mRNA and ε germ-line gene transcripts was suppressed by fluticasone propionate treatment. Our results suggest that local IgE synthesis and cytokine regulation of heavy chain switching to IgE occur in the nasal mucosa.

1 Introduction

1.1 Background

Immunoglobulin E (IgE) mediates allergic reactions by binding to its high-affinity receptor, FcεRI, on mast cells and basophils. Cross-linking of the attached IgE by multivalent antigen then triggers the allergic response. IgE antibodies on the mast cells of the nasal mucosa are cross-linked by airborne allergens, leading to the symptoms of hayfever. The provenance of the IgE on nasal mast cells (and indeed on mast cells at other mucosal surfaces, such as the lung, gut and skin) is unknown [1]. IgE may be secreted by B cells and bound to receptors on mast cell precursors and basophils elsewhere and transported to the nasal mucosa, or it may be produced by B cells within the nasal mucosa itself. IgE is the least abundant class of Ab (~ 100 ng/ml in normal serum, compared to 10 mg/ml for IgG). The critical step in the commitment of B cells to the synthesis of IgE is referred to as heavy chain switching, and comprises somatic recombination within the immunoglobulin heavy chain gene cluster to transform the ε germ-line gene into the rearranged gene. This then gives rise to mRNA for the ε heavy chain of IgE [2]. If B cells do provide a local source of IgE in the nasal mucosa, then there are two further possibilities. B cells may switch to IgE before or after they migrate to the tissue.

Evidence in favor of local IgE synthesis has recently come from studies of cultured B cells derived from human nasal secretions, which express IgE at both the mRNA and the protein level [3–5]. It is not clear, however, that these cells ever resided in the nasal mucosa. Heavy chain switching occurs in B cells within lymphoid tissue *in vivo* [6, 7] and in isolated B cells *in vitro*. It is not known whether B cells within non-lymphoid tissues ever undergo switching.

Two signals are required for heavy chain switching to IgE both *in vitro* and *in vivo*. IL-4 and the recently described IL-13 are agents that target the ε gene for recombination; cross-linking of CD40 on the B cell membrane then initiates recombination to proceed in a proportion of the cells [8]. IL-4 is released from cytoplasmic stores on activation of mast cells [9] and, like IL-13 [10], is secreted by a subset of T helper cells (Th2 cells) [11]. CD40 ligand (CD40L) is constitutively expressed by tissue mast cells and induced on T cells, following activation by antigen [12, 13]. IL-4 is required for the differentiation of T cells into the Th2 phenotype, which secrete their own IL-4, and thereafter autocrine production of IL-4 supports cell proliferation [14]. Thus it has been inferred that activation of mast cells by allergen may promote the differentiation of T cells into Th2 cells and initiate switching to IgE in local B cells [12, 15–18]. The resulting Th2 cells, upon activation by antigen, could then continue the process.

We observed earlier that allergen provocation in the nasal mucosa of hayfever patients resulted in early (0–60 min) and late (1–24 h) nasal symptoms, accompanied by the recruitment of Th2 cells (predominantly $CD4^+$ Th2 lymphocytes) expressing IL-4 [19, 20] and IL-13 [21], assessed

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Abbreviation: **FP:** Fluticasone propionate

Key words: IgE / Heavy chain switching / Allergic rhinitis or hayfever / Corticosteroid

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The Transcription Factor B Cell-Specific Activator Protein (BSAP) Enhances Both IL-4- and CD40-Mediated Activation of the Human ε Germline Promoter[1]

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Induction of isotype switching to a particular C_H gene correlates with the transcriptional activation of the same gene in germline configuration. Induction of correctly spliced germline transcripts is necessary to target a switch region for recombination and switching. Different cytokines activate transcription at different germline promoters. Because binding sites for the B cell-specific transcription factor BSAP are located upstream of several switch regions in the Ig locus, BSAP might play a role in isotype switching by regulating germline transcription. We investigated whether BSAP plays a role in the transcriptional regulation of the ε germline promoter in human B cells. We identified human EBV-negative B cell lines that express ε germline transcripts upon stimulation with IL-4. Electrophoretic mobility shift assay analysis showed that the human ε germline promoter binds BSAP. BSAP activity was expressed constitutively and was not affected by stimulation with IL-4 and/or anti-CD40 mAb. Reporter assays with constructs containing a luciferase gene driven by the ε germline promoter, with or without mutations in the BSAP binding site, showed that BSAP plays a role in both IL-4-dependent induction and CD40-mediated up-regulation of human ε germline transcription. Furthermore, ε germline promoter activity was abrogated in REH cells that express a BSAP polypeptide truncated in the *trans*-activation domain. Among the transcription factors that regulate ε germline expression, BSAP is unique, in that it is B cell-specific and is at the merging point of two signaling pathways that are distinct but both critical for the induction of IgE switching. *The Journal of Immunology,* 1997, 158: 5874–5882.

Isotype switching results from a DNA recombination event that juxtaposes different downstream C_H genes to the expressed VDJ gene, thus changing the effector function, but not the specificity, of the Ab molecule. Isotype switching is not a random event, but is directed by cytokines in conjunction with the regulation of B cell proliferation and differentiation (reviewed in Refs. 1 and 2). Molecular analysis has shown that cytokine-dependent induction of isotype switching to a particular C_H gene almost invariably correlates with the transcriptional activation of the same gene in germline configuration. Several murine and human germline transcripts have been cloned and share structural similarities. The germline transcripts initiate from TATAA-less promoters a few kilobases upstream of the switch region and proceed through short exons (I_H exons), the switch regions, and C_H exons. The I_H exon is then spliced to the first exon of the C_H gene. During recombination, the region containing the germline promoter and the I_H exon is deleted as part of a switch circle (3, 4).

Induction of correctly spliced transcripts is thought to be necessary to target the appropriate switch region for recombination and switching (5–7). Therefore, it is important to understand how the induction of germline transcripts is regulated. Different cytokines specifically activate transcription at the appropriate germline promoter. In particular, IL-4 has been shown to induce ε germline transcripts in murine (8–10) and human (11, 12) B cells. IL-4-dependent ε germline transcription in B cells is strongly up-regulated upon CD40 engagement (13). This transcriptional effect of CD40 may be critical for switching (14), because optimal levels of transcription through the switch region may be required to target recombination.

Several *cis*-acting elements have been characterized that regulate IL-4-induced transcription. A highly conserved DNA sequence 5′ to the major initiation sites for murine germline ε RNA has been shown to contain an IL-4-responsive region that binds three transcription factors: a member of the STAT family, STAT6, one or more members of the C/EBP[3] family, and NF-κB/p50 (15). Mutation of any of the binding sites for these factors abolished or reduced the IL-4 inducibility of the ε promoter (15). A binding site for STAT6 has been identified in the human ε germline promoter as well (16–18).

A region in the murine ε germline promoter 3′ to the STAT6 binding site has been shown to bind a constitutively expressed transcription factor, B cell lineage-specific activator protein (BSAP) (19). BSAP is encoded by Pax5, a member of the Pax gene family of homeodomain transcription factors, and is the mammalian homologue of the sea urchin tissue-specific transcription activator protein, a regulator of late histone genes. BSAP is

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[3] Abbreviations used in this paper: NF-κB, nuclear factor-κB; BSAP, B cell lineage-specific activator protein; C/EBP, C/enhancer-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; COUP, chicken ovalbumin upstream promoter; luc, luciferase; RSV, Rous sarcoma virus; β-gal, β-galactosidase; RT-PCR, reverse transcription-polymerase chain reaction.

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expressed in pro-B, pre-B, and mature B lymphocytes, but not in terminally differentiated plasma cells (20). BSAP knockout mice show a complete block of early B cell differentiation at the pro-pre-BI stage (21). Binding sites for BSAP have been identified in the promoter of several B cell-related genes such as CD19 (22), the surrogate light chains λ5 and VpreB1 (23), X box binding protein 1 (24), the tyrosine kinase *blk* (25), as well as upstream of several Ig switch regions (μ, γ1, γ2, ε, and α) and in the control region located 3′ of Cα (26, 27). Interestingly, BSAP contributes to the activity of the CD19 promoter (22), but has a negative effect on the Ig 3′ α enhancer (26, 27) and on the promoters of the X box binding protein 1 (24) and J chain (28) genes. A deregulation of Pax5 transcription due to translocation of the Eμ enhancer of the IgH locus next to the Pax5 promoter has been recently proposed to contribute to the pathogenesis of small lymphocytic lymphomas with plasmacytoid differentiation (29).

Conflicting reports exist about the role of BSAP in the regulation of ε germline transcription in murine B cells. Mutation (10) or deletion (19) of the BSAP binding site abrogated induction of ε germline promoter activity in B cells stimulated with IL-4 and LPS. Activity was restored by the introduction of a BSAP binding site from the sea urchin histone gene H2A-2.2 (19). In contrast, deletions in the BSAP binding region were unable to affect the inducibility of the promoter in cells stimulated with IL-4 but not LPS (15). We investigated whether BSAP plays a role in the transcriptional regulation of the ε germline promoter in human B cells. Our data show that BSAP binds to the human ε germline promoter and is essential for both IL-4-dependent induction and CD40-mediated up-regulation of human ε germline transcription.

Materials and Methods

Cell lines and cultures

The EBV-negative, sIgM$^+$ B cell lines BL-2 and BJAB were provided by Dr. G. Aversa (DNAX Research Institute, Palo Alto, CA) and Dr. E. Kieff (Harvard Medical School, Boston, MA), respectively. The cell line REH was originally established from a patient with acute lymphoblastic leukemia (30). The T cell line Jurkat was obtained from American Type Culture Collection (Rockville, MD). All cell lines were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Biological Industries, Kibbutz Beit Haemek, Israel). For stimulation, B cells were incubated for 48 h with rIL-4 (provided by Dr. J. E. de Vries, DNAX Research Institute) at 100 U/ml and/or with anti-CD40 mAb 626.1 (a gift from Dr. S. M. Fu, University of Virginia, Charlottesville, VA) at 5 μg/ml. Preliminary experiments had shown that these concentrations resulted in optimal induction of ε germline transcripts.

RNA isolation and semiquantitative reverse transcription (RT)-PCR

RNA was isolated from B cells as described by Chomczynski and Sacchi (31), and cDNA was synthesized and subjected to semiquantitative PCR as previously described (32). For each PCR reaction, one primer was 5′ labeled and added in a 1:10 molar ratio with cold primer. ε germline transcripts were detected as a 409-bp band amplified by primers corresponding to the 3′ end of human Iε (5′-GACGGGCCACACCATCCACAGGCAC CAAATGGACGAC, nucleotide +93/+129 in Fig. 1*B*) and the 5′ end of Cε2 (5′-CAGGACGACTGTAAGATCTTCACG, nucleotide 1754–1777, GenBank accession No. J00222). As a control, a 438-bp band corresponding to GAPDH transcripts was amplified using appropriate primers (5′-GGGAAGGTGAAGGTCGGAGTC and 5′-CTGATGATCTTGAGGCTG TTG, nucleotides 1456–1476 and 3818–3848, GenBank accession no. J04038). PCR was performed on a thermocycler (Hybaid, Teddington, U.K.) for 25 cycles (1 min each at 94, 65, and 74°C) for ε germline transcripts and for 15 cycles (1 min each at 94, 60, and 74°C) for the GAPDH control. The number of amplification cycles was such that the maximum signal intensity for a set of samples was within the linear portion of a product vs the template amplification curve.

Oligonucleotides and probes for electrophoretic mobility shift assays (EMSA)

The −24/+12 probe was generated by isolating a single-stranded template by asymmetric PCR with the upper strand (5′-TTAGCTGAAAGCACTG AGGCAGAG, nucleotide −24/−1 in Fig. 1*B*) at 1 μM and the lower strand (5′-GGGTAGGGGGAGCTCT, nucleotide −4/+5) at 0.05 μM. The −24/+64 probe was generated by producing a single-stranded template by asymmetric PCR with the upper strand (nucleotide −24/−1, as above) at 1 μM and the lower strand (5′-CCCAGATGATCAGTAACCG TG, nucleotide +44/+64) at 0.05 μM. The second strand was synthesized with *Taq* polymerase in the presence of [α-^{32}P]dATP and the lower strand oligonucleotide (1 μM).

For competition, oligonucleotides were synthesized that correspond to known BSAP binding sites in the murine 5′ Sγ2a region (NF-HB; 5′-GATCAGAATTGTGAAGCGTGACCA) and in the histone H2A-2.2 promoter (5′-TGTGACGCAGCGGTGGGTGACGACT) (19, 26). Mutant BSAP competition oligonucleotides were generated by PCR by using as forward primers BSAP1XMUT (5′-TTAGCTGAAAGCACTGAGG*A*AG AG) and BSAP2XMUT (5′-TTAGCTGAAAGCACTGAG*TA*AGAG; nucleotide −24/+12 in Fig. 1*B*) and as reverse primer 5′-CCCAGATGAT CAGTAACCGTG (nucleotide +44/+64 in Fig. 1*B*) for both mutants. An unrelated oligonucleotide (chicken OVA upstream promoter (COUP); 5′-TCGACTCTATGGTGTCAAAGGTCAAACTTCTGAC) was used as a control. All oligonucleotides were obtained from PRIMM (Milan, Italy). Competition oligonucleotides were annealed in 100 mM NaCl, 10 mM Tris (pH 8), and 0.1 mM EDTA by heating to 95°C for 10 min and cooling slowly to the annealing temperature. All probes and competitors were purified on polyacrylamide gels.

Preparation of nuclear extracts

Cells (2–3 × 10^6) were washed in PBS and resuspended in 400 μl of buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT) with protease inhibitors (PMSF (0.2 mM), aprotinin (1 μg/ml), and antipain, pepstatin, and leupeptin (all at 3 μg/ml)) and phosphatase inhibitors (1 mM benzamidine, 1 mM orthosodium vanadate, 1 mM sodium fluoride, and 5 mM β-glycerol phosphate). After a 10-min incubation on ice, the release of nuclei was judged by trypan blue staining. The nuclei were sedimented in a microcentrifuge for 12 s at 4°C and then resuspended in 2 pellet volumes of buffer C (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM DTT, with protease and phosphatase inhibitors). The total volume of the nuclei was measured, and the nuclei were transferred to a new tube. One volume of buffer C containing 840 mM NaCl was then added, and the nuclei were extracted for 20 min on ice, with occasional mixing. The preparation was sedimented in a microcentrifuge for 5 min at 4°C, and the extracts were frozen on dry ice and stored at −70°C. Protein concentrations were determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL).

EMSA

Nuclear extracts (5 μg) were added to binding buffer (10 mM Tris (pH 8), 0.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT; 20 μl), with poly (dI-dC) (Pharmacia, Uppsala, Sweden; 5 μg) and ^{32}P-labeled probe (5 fmol) in the presence or the absence of competitors. NaCl and glycerol were adjusted to 0.1 M and 10%, respectively. After a 30-min incubation on ice, the binding reactions were run on a 4% polyacrylamide gel in 50 mM Tris, 50 mM boric acid, 1 mM EDTA (pH 8), and 4% glycerol at 30 mA for 2 to 5 h at 4°C. Supershifts were performed by preincubating the nuclear extracts with the appropriate antisera (0.25 μl/reaction) for 30 min on ice before adding the probe and competitors. Polyclonal rabbit antisera against human BSAP were previously described (20). One antiserum is directed against the paired domain of BSAP (amino acids 17–145) and blocks DNA binding; the other is directed against C-terminal sequences (amino acids 189–391) and causes supershifting in EMSA. A polyclonal antiserum against the human transcription factor HOXB3 was a gift from Dr. G. Viale (San Raffaele Scientific Institute, Milan, Italy).

ε germline promoter reporter constructs

Figure 1*A* shows the map of the human Iε exon and Cε locus. Figure 1*B* shows part of the genomic sequence contained in the ε germline promoter construct. Position −291 (not shown) corresponds to the A in the *Rsa*I site (nucleotide 241, GenBank accession No. X56797) used to clone the region in the reporter vector. To obtain the human ε germline promoter construct, a 422-bp fragment (−291/+131) that encompasses the promoter (17) and

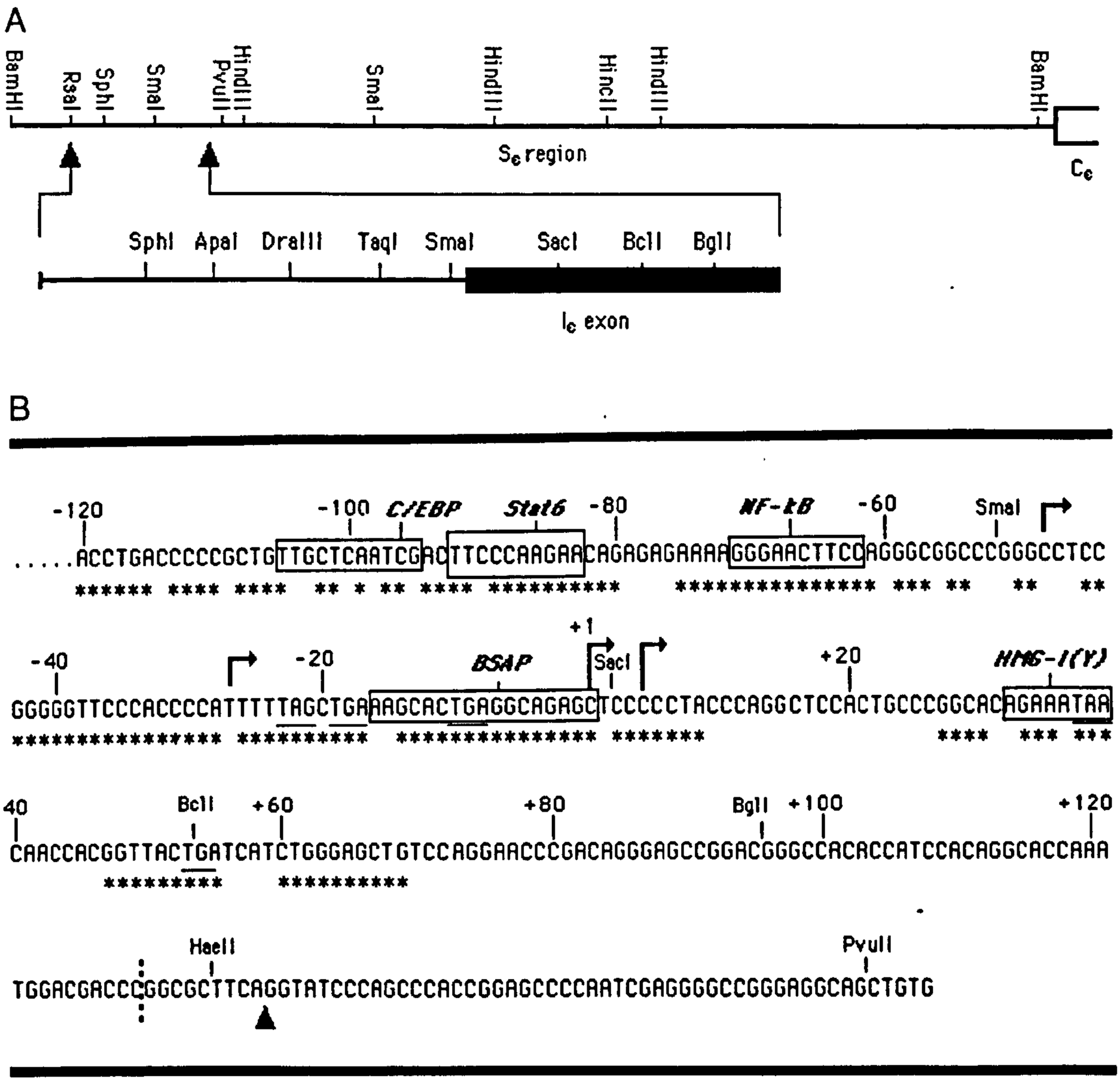

FIGURE 1. Map of the human germline ϵ gene and ϵ transcripts. *A*, Restriction map of the unrearranged Cϵ gene, with the Iϵ exon and the Sϵ region. The region contained in the GL-luc reporter construct is indicated by arrows. *B*, Part of the genomic sequence contained in the germline ϵ promoter construct. Position −291 (not shown) corresponds to the A in the *Rsa*I site (nucleotide 241, GenBank accession No. X56797) used to clone the region in the pGL3 reporter vector. The major transcription start sites in BL-2 and BJAB cells were determined by primer extension and are indicated by arrows. Position +1 corresponds to the major ϵ RNA start site in BL-2 cells. The 3′ end of the construct is marked by a dashed vertical line. The splice site for Cϵ1 is indicated by an arrowhead. The binding sites for transcription factors identified in the murine (10, 15, 19, 42, 55) and human (16, 17, 18, 46, 58) ϵ germline promoter are boxed. The asterisks mark the evolutionarily conserved sequence, a segment highly (>80%) homologous between human and murine sequences. Stop codons are underlined.

ends 9 bp upstream of the splice site for Cϵ, was inserted into the promoterless pGL3 basic vector (Promega, Madison, WI), that contains a luciferase (luc) reporter gene. Because of the discrepancies between the sequences published for the region containing the human ϵ germline promoter (11, 33), we used PCR with Pfu DNA polymerase (Stratagene, La Jolla, CA) to amplify this region from genomic DNA of three nonatopic donors. The forward primer was 5′-CCTGGGAGTGAGTACAA GGTGAG, and the reverse primer was 5′-GGTGGGCTGGGATACCT GAAG (nucleotides 25–47 and 661–681 of GenBank accession No. X56797). The PCR product was digested with *Rsa*I and *Hae*II. The 3′ end was made blunt and ligated into the *Sma*I site of pGL3, thus obtaining the ϵ GL-luc construct. The insert and the junctions were sequenced by the dideoxynucleotide chain termination/extension method (34) using the Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH). Two clones from each of the three donors were fully sequenced. All six sequences were identical, except at position +97 (Fig. 1*B*), which was either a G or an A. This probably represents a polymorphism. The reporter activity of the luc constructs containing either the G or the A was identical (data not shown).

The deletion mutant lacking the BSAP binding site (BSAP del-luc) was generated by PCR assembly of two fragments, using the ϵ GL-luc construct as the template for amplification. A 274-bp PCR product was obtained, using as forward primer 5′-CACCCCATTTTTAGCTCCCA GGCTCCACTGC, which corresponds to nucleotide −34/+24 in Figure 1*B* and introduces a 27-bp deletion (nucleotide −18/+9). The reverse primer was 5′-GCAGTTGCTCTCCAGCGGTTC (GL4 primer, Promega), which maps to position 146–165 in pGL3. The other PCR product of 357 bp was generated using as forward primer 5′-CTAGCAAA ATAGGCTGTCCC (RVp3 primer, Promega), which maps to position 4761–4780 in pGL3, and as reverse primer 5′-AGCTAAAA ATGGGGTG, which maps to position −34/−19 in the insert. The two PCR products were assembled by a second round of amplification using primers RVp3 and GL4, then digested with *Kpn*I and *Hin*dIII and directionally cloned into pGL3.

The BSAP mut1-luc construct was generated using as forward primer RVp3 and as reverse primer 5′-GGGAGCTCT*T*CCTCAG (nucleotide −11/+5 in Fig. 1*B*), where a G is replaced by a T. The BSAP mut2-luc

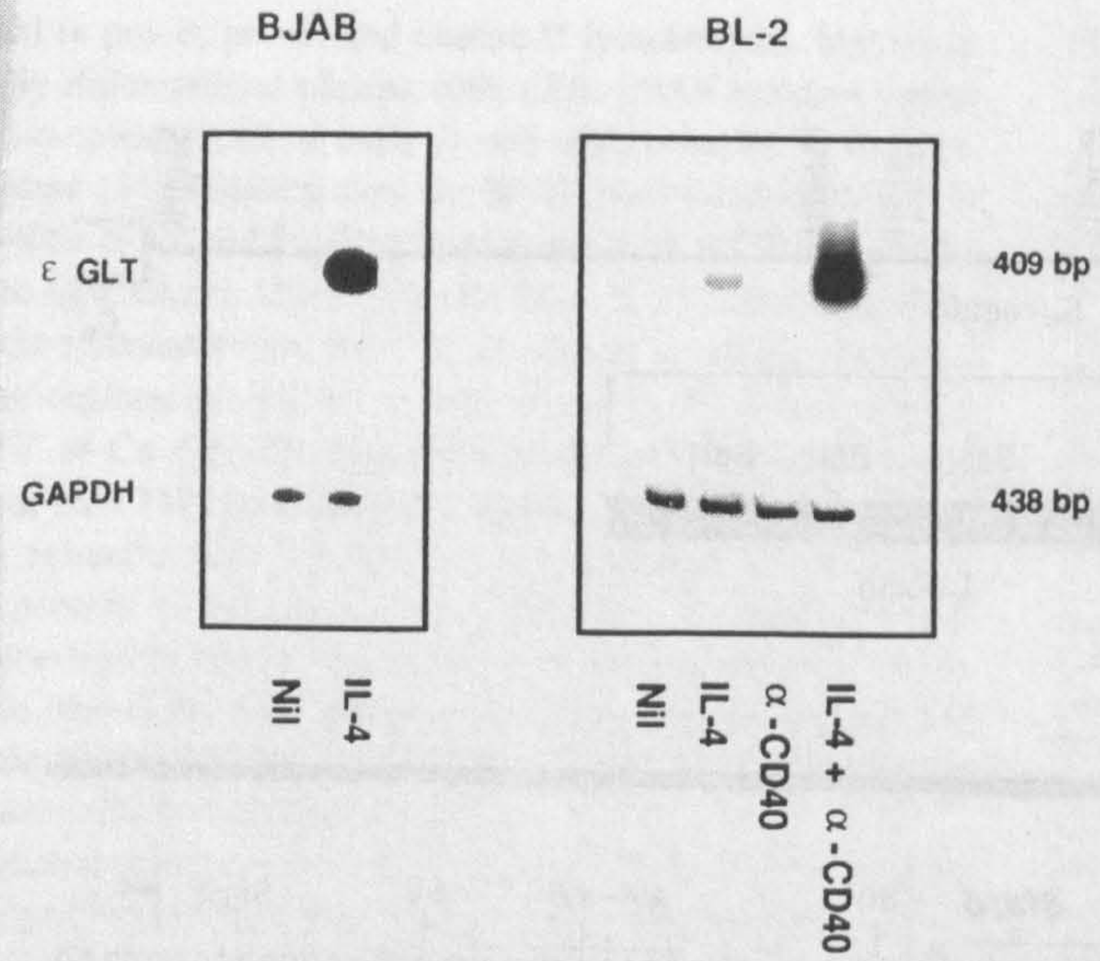

FIGURE 2. IL-4 induces ϵ germline transcripts in human lymphoblastoid B cells. BL-2 and BJAB cells were treated with IL-4 (100 U/ml) and/or anti-CD40 mAb (5 μg/ml) for 48 h, and then assessed for levels of ϵ germline transcripts by semiquantitative RT-PCR. PCR was performed for 25 cycles for ϵ germline transcripts and for 15 cycles for the GAPDH control.

construct was obtained using as reverse primer 5′-GGGAGCTCT*TA*CT CAG, where GC is replaced by TA. The PCR products were digested with *Sac*I and inserted in the *Sac*I sites of ϵ GL-luc. The deletion and the mutations were confirmed by sequence analysis. All plasmids were prepared for transfection by two rounds of purification on CsCl density gradients.

Transfections and reporter assays

Lymphoblastoid B cells (10×10^6) were transiently cotransfected with luc constructs (10 μg) and a control plasmid (Rous sarcoma virus (RSV)-β-galactosidase (β-gal); 6 μg) by treatment with DEAE-dextran (Pharmacia; 0.5 mg/ml) for 30 min at 37°C. A luc plasmid (4 μg) driven by the RSV promoter and enhancer was used as a positive control in each set of transfections. After transfection, the cells were split into aliquots and cultured in the presence or the absence of IL-4 (100 U/ml) and anti-CD40 mAb 626.1 (5 μg/ml). Each transfection was performed in duplicate. After 48 h, cell extracts were prepared, and luciferase activity was determined according to the Luciferase Assay System (Promega) protocol. The same cell extracts were used to determine β-gal activity using the chemiluminescent Galacto-Light kit (Tropix, Bedford, MA) according to the manufacturer's instructions. All readings were taken using a Lumat 9501 luminometer (Berthold, Wildbach, Germany). The results obtained for each transfection were normalized to β-gal activity and expressed as relative luciferase activity. The values obtained for mock-transfected cells (<100 light units for luciferase and <200 light units for β-gal) were subtracted as background.

Results

IL-4 induces ϵ germline transcripts in human B cell lines

A number of human B cell lines were initially screened by semiquantitative RT-PCR for the expression of ϵ germline transcripts upon IL-4 stimulation (data not shown). Among those that expressed ϵ germline transcripts, two EBNA-negative lines, BL-2 and BJAB, were chosen for subsequent studies. Indeed, EBV-encoded protein(s) provides B cells with a signal(s) that triggers IgE switching (12, 35), possibly by interfering with the CD40 signaling pathway (36, 37). Figure 2 shows that ϵ germline transcripts were undetectable in unstimulated BL-2 and BJAB cells, but were readily induced following a 48-h incubation with IL-4 (100 U/ml). As expected (13), ϵ germline transcription was not induced by anti-CD40 mAb alone (5 μg/ml), but was strongly up-regulated when CD40 was engaged in the presence of IL-4 (Fig. 2, *right panel*).

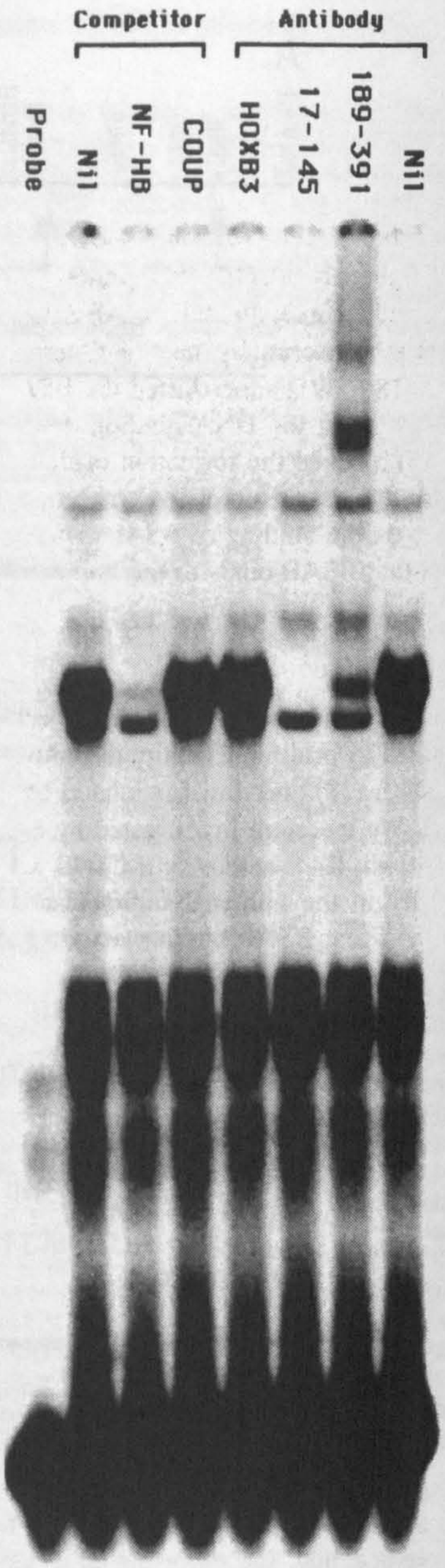

FIGURE 3. BSAP binds to the human ϵ germline promoter. EMSA were performed using nuclear extracts from unstimulated BJAB cells and a probe (−24/+12) that spans the BSAP binding site in the ϵ germline promoter. Nuclear extracts were preincubated with rabbit Abs (0.25 μl/reaction) specific for the paired (DNA binding) domain of BSAP (amino acids 17–145), the C-terminal region of the protein (amino acids 189–391), or human HOXB3 for 30 min on ice before adding the probe and the competitors. Competitors (NF-HB and COUP) were added at a 100-fold molar excess. The arrow indicates the band corresponding to the BSAP complex.

BSAP binds to the human ϵ germline promoter

To assess whether BSAP binds to the human ϵ germline promoter, EMSA analysis was performed using a probe (nucleotide −24/+12 in Fig. 1*B*) that spans the region homologous to the BSAP binding site identified in the murine ϵ promoter (19). Figure 3 shows that nuclear extracts from unstimulated BJAB cells contain a constitutively expressed protein that retards the migration of the BSAP probe. The formation of the DNA-protein complex was specifically competed by a BSAP site located upstream of the murine

Sγ2a region (NF-HB) and by the BSAP site in the sea urchin histone H2A-2.2 promoter (data not shown), but not by an unrelated oligonucleotide (COUP). An identical EMSA pattern was observed using nuclear extracts from unstimulated BL-2 cells (data not shown). Consistent with the B cell specificity of BSAP expression (20), the BSAP complex was undetectable in nuclear extracts from Jurkat T cells (data not shown).

To unambiguously identify the protein that binds the BSAP probe, we tested the effect of anti-BSAP antisera in EMSA. Figure 3 shows that an antiserum against the C-terminal region of BSAP (amino acids 189–391) supershifted the BSAP complex, whereas an antiserum against the DNA binding domain of BSAP (amino acids 17–145) blocked the formation of the complex. In contrast, an antiserum against an unrelated transcription factor (HOXB3) had no effect on the binding of BSAP. These results conclusively demonstrate that BSAP binds to the human ϵ germline promoter.

BSAP binding activity in human B cells is not regulated by IL-4 and/or CD40 engagement

BSAP binding activity in splenic murine B cells has been shown to be up-regulated by proliferative stimuli (mitogens, cross-linking of sIgD, or CD40) (38) and down-regulated by OX40 ligand cross-linking (39). We therefore investigated by EMSA whether a 48-h stimulation with IL-4 and/or anti-CD40 mAb could modulate BSAP activity in the human lymphoblastoid cells under study. Figure 4 shows that BSAP binding activity, as defined by use of the BSAP-specific competitor NF-HB, was comparable in nuclear extracts from unstimulated and stimulated BL-2 cells. In contrast, vigorous induction of ϵ germline transcripts was observed in the same experiments (data not shown). These results indicate that BSAP binding activity is not regulated by IL-4 and/or CD40 engagement.

The highly conserved C in the BSAP site is essential for DNA/protein interactions

A cytosine residue (nucleotide −5 in Fig. 1*B*) in the BSAP consensus sequence recently identified is highly conserved in the known BSAP binding sites (40, 41). A point mutation (C→A) at this site has been shown to be sufficient to strongly impair the binding of BSAP to the sea urchin tissue-specific transcription activator protein site (20). In preparation for subsequent functional studies, EMSA were performed to assess whether a DNA fragment containing the C→A mutation could efficiently compete binding of human BSAP to a probe (nucleotide −24/+64) containing the wild-type sequence. Figure 5 (*left panel*) shows that the wild-type sequence competed BSAP binding partially when used at a 10-fold molar excess and almost completely when used at a 30-fold molar excess. Introduction of the C→A mutation (mut 1) strongly reduced competition over the whole molar excess range. A G residue 1 base 5′ to the mutated C is also contained in the BSAP consensus sequence (40). When the conserved G residue was mutated to a T in addition to the C→A mutation (mut 2), no further reduction in competition was observed. Thus, the conserved C in the BSAP site is essential for in vitro DNA/protein interactions. This conclusion was further supported by the finding that a −24/+12 BSAP probe containing the C→A mutation (mut 1) was unable to bind BSAP in EMSA (Fig. 5, *right panel*).

BSAP regulates both the IL-4-dependent induction and the CD40-mediated up-regulation of ϵ germline transcription

To functionally characterize the role of BSAP in the regulation of human ϵ germline transcription, reporter assays were performed using constructs that contain a region encompassing the human ϵ germline promoter (17), with or without mutations in the BSAP

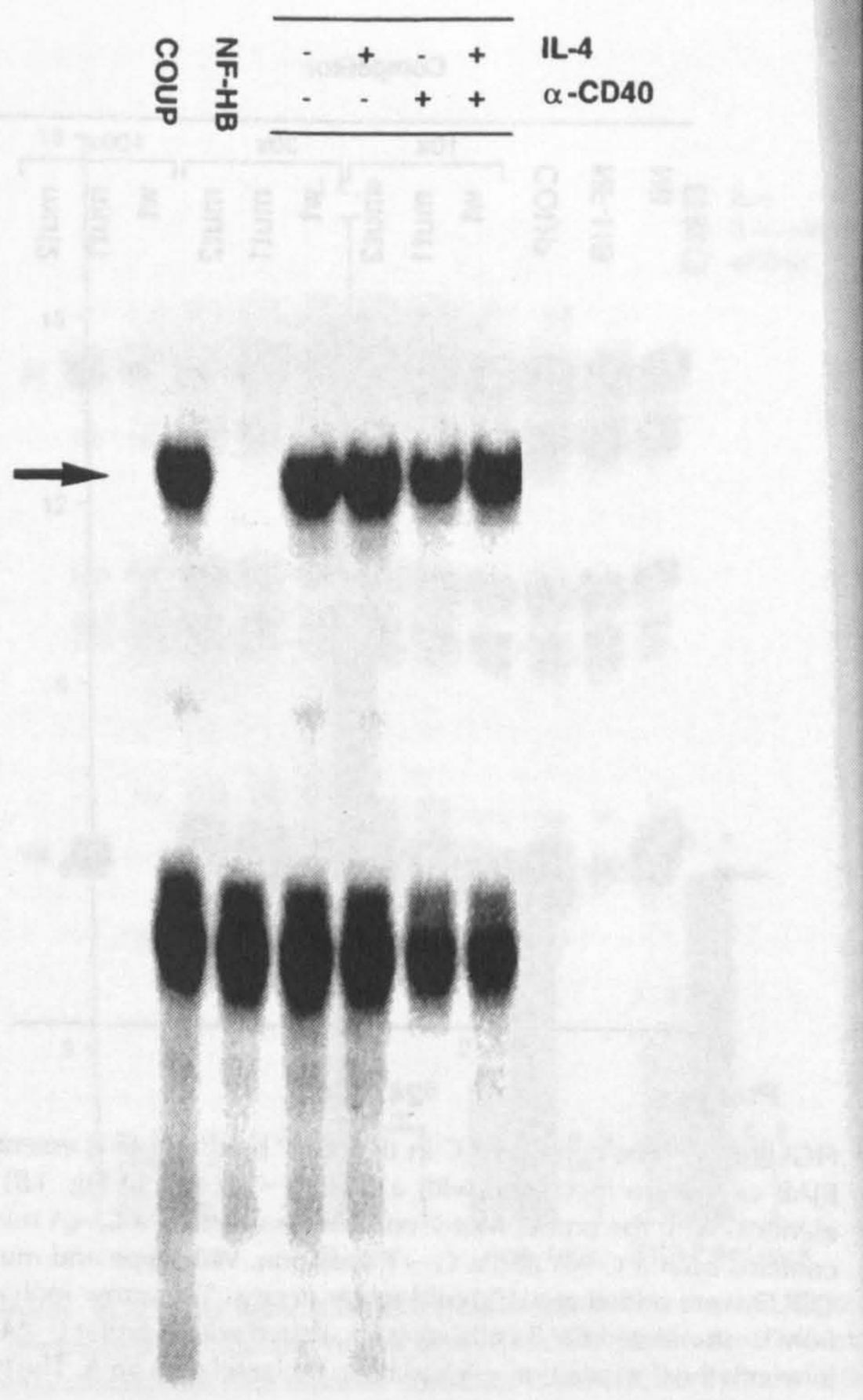

FIGURE 4. BSAP binding activity is not regulated by IL-4 and/or CD40 engagement. EMSA were performed using a probe (−24/+12) that spans the BSAP binding site in the ϵ germline promoter, and nuclear extracts from BL-2 cells incubated for 48 h in the presence of medium, IL-4 (100 U/ml), and anti-CD40 mAb (5 μg/ml), alone or in combination. Competitors (NF-HB and COUP) were added at a 100-fold molar excess. The arrow indicates the band corresponding to the BSAP complex.

binding site. The human GL-luc construct was obtained by inserting a 422-bp fragment of the promoter into the promoterless pGL3 luc vector. The BSAP mut 1-luc and BSAP del-luc constructs contained the C→A mutation at nucleotide −5 (Fig. 1*B*) and a 27-bp deletion encompassing the BSAP binding site (nucleotide −18/+9), respectively. The reporter constructs were transiently transfected into the lymphoblastoid B cell lines; after transfection, the cells were divided into aliquots and incubated in the presence or the absence of IL-4 and anti-CD40 mAb. Luciferase activity was assessed 48 h later. An RSV-β-gal plasmid was always cotransfected with the reporter constructs as an internal control for variations in transfection efficiency.

Preliminary experiments indicated that expression of the luc constructs was inducible at higher and more consistent levels in BL-2 than in BJAB cells (data not shown). BL-2 cells were therefore used for this set of studies. Figure 6 (*right panel*) shows that IL-4 stimulation of BL-2 cells transfected with the construct containing the intact promoter (GL-luc) resulted in a 4.9-fold induction of luciferase activity. Transcription driven by the ϵ germline promoter was enhanced modestly by anti-CD40 mAb, but was

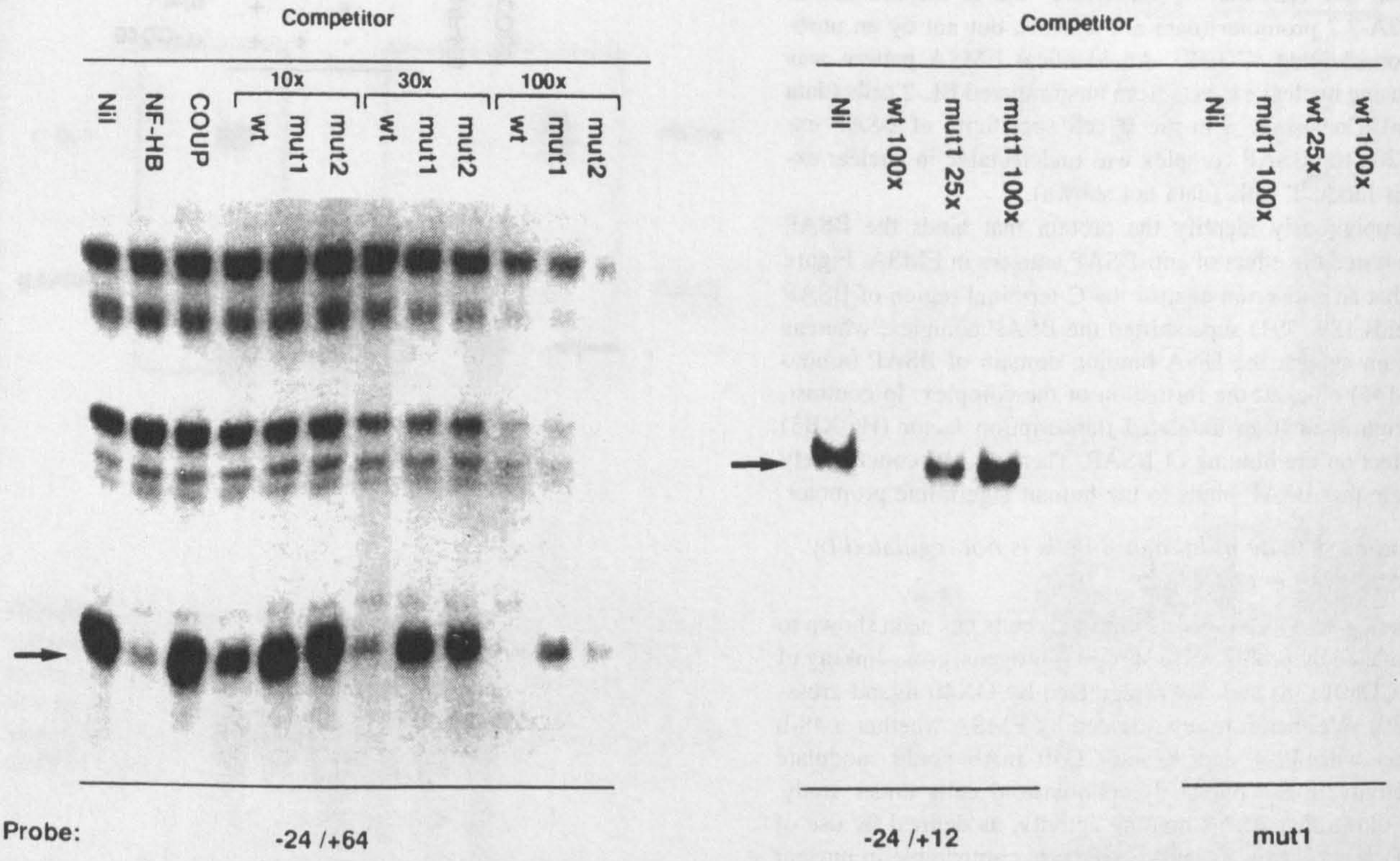

FIGURE 5. The conserved C in the BSAP binding site is essential for DNA/protein interactions. *Left panel,* Nuclear extracts from unstimulated BJAB cells were incubated with a probe (−24/+64 in Fig. 1*B*) containing the wild-type (WT) BSAP binding site. The wild-type competitor is identical with the probe. Mut 1 competitor contains a C→A mutation at the highly conserved C at position −5 in the BSAP binding site. Mut 2 contains both a C→A and a G→T mutation. Wild-type and mut competitors were added at 10-, 30-, and 100-fold molar excesses. NF-HB and COUP were added at a 100-fold molar excess. The arrow indicates the band corresponding to the BSAP complex. *Right panel,* Nuclear extracts from unstimulated BJAB cells were incubated with a probe (−24/+12) corresponding to the wild-type BSAP binding site or with the mut 1 probe, in which the C at position −5 had been replaced with an A. The two oligonucleotides were also used as competitors and added at 25- and 100-fold molar excesses.

up-regulated 14.9-fold when IL-4 and anti-CD40 mAb were added in combination. The introduction of the C→A mutation at position −5 in the BSAP binding site (BSAP mut 1-luc) reduced the IL-4 inducibility of the promoter by >50% and the response to the combination of IL-4 and anti-CD40 mAb by 65% even though the synergism between the two signals was preserved. Deletion of the entire BSAP binding site (BSAP del-luc) had comparable effects. These results indicate that BSAP regulates both the IL-4-dependent induction and the CD40-mediated up-regulation of ϵ germline transcription in human B cells. Notably, the deletion and the mutation introduced in the BSAP site did not affect the basal expression of the reporter constructs in BL-2 cells (Fig. 6, *left panel*). Thus, unlike the elements that bind STAT6 and HMG-I(Y), which are involved in both activation and repression of the ϵ promoter (17, 42), the region that binds BSAP seems to be involved only in activation.

ϵ germline promoter activity is abrogated in REH cells that express a BSAP protein truncated in the trans-activation domain

It has been recently shown that the human acute lymphoblastic leukemia cell line REH carries a frameshift mutation in one Pax5 allele that results in premature termination of translation and truncation of the *trans*-activating domain of BSAP. Thus, REH cells synthesize both full-length BSAP and a truncated polypeptide that lacks the *trans*-activating function (43). Interestingly, transcripts for CD19, a BSAP target gene, are strongly reduced in REH cells compared with most other human B cell lines (22). The lower levels of CD19 mRNA are thought to reflect either the lower level of *trans*-activation-competent BSAP and/or negative interference by the mutated protein. Because of these peculiar properties, we used the REH cell line as an additional model to characterize the role of BSAP in the activation of the ϵ germline promoter. To this purpose, REH cells (and BL-2 cells, as a control) were transiently transfected with the GL-luc reporter construct and subsequently stimulated with IL-4 in the presence or the absence of anti-CD40 mAb. No ϵ promoter activity was detected in REH cells, whereas, as expected (see Fig. 6), a vigorous response was elicited in BL-2 cells (data not shown). Furthermore, RT-PCR analysis failed to reveal expression of endogenous ϵ germline transcripts in REH cells treated with IL-4 and anti-CD40 mAb (data not shown).

While the full extent of the functional defect in REH cells remains to be established, our results suggest that a functional BSAP protein is essential for activation of the ϵ germline promoter not only within an artificial reporter construct, but also in its natural chromatin context. We are currently testing the effects of BSAP overexpression on the phenotype and function of REH cells.

Discussion

Binding sites for BSAP are located upstream of several switch regions in the Ig heavy chain gene cluster (19, 44, 45). This finding led to the hypothesis that BSAP might play a role in isotype switching by regulating germline transcription, a process known to be a prerequisite for switch recombination. However, the evidence gathered to date is controversial. BSAP was found to be necessary

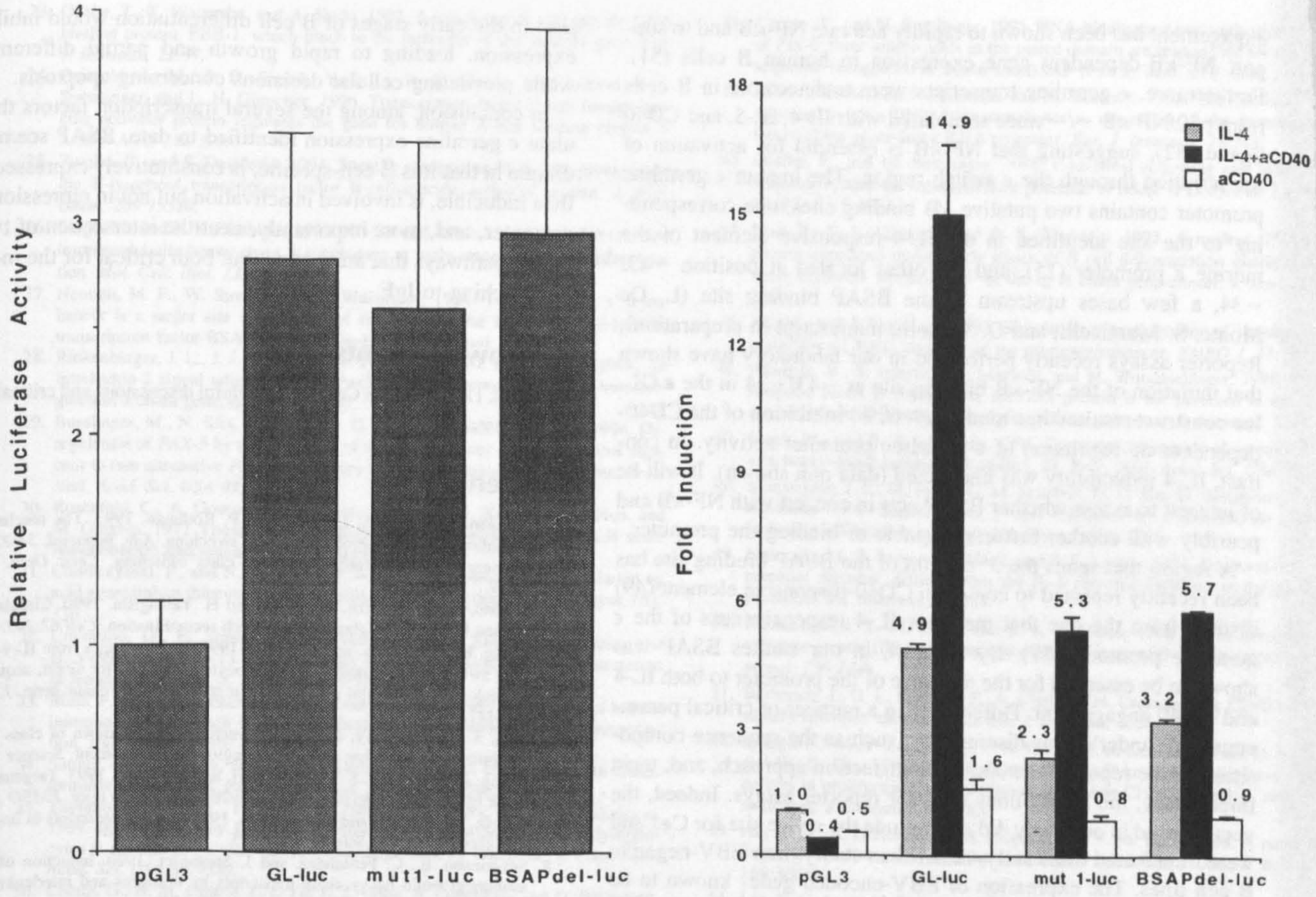

FIGURE 6. Results of reporter assays with ϵ germline promoter luc constructs. BL-2 cells were transiently transfected with the luc reporter constructs using DEAE-dextran and split into aliquots that were incubated in the presence or the absence of IL-4 (100 U/ml) and anti-CD40 mAb (5 μg/ml). Luc activity was assessed 48 h later. A β-gal control plasmid was cotransfected with the reporter construct to normalize for transfection efficiency. Values obtained in mock-transfected cells were subtracted as background. *Left,* Basal expression of luc reporter construct in BL-2 cells. Results are reported as relative luciferase activity after subtraction of background (mock transfection). *Right,* Expression of the reporter constructs in BL-2 cells stimulated with IL-4 (100 U/ml) and/or anti-CD40 mAb (5 μg/ml) for 48 h. Fold induction represents the ratio of luc activity (normalized for β-gal activity) between treated and untreated cells. The results show the mean ± SE of three independent experiments, each performed in duplicate.

for activation of the ϵ germline promoter in murine B cells stimulated with LPS and IL-4 (10, 19). In contrast, in two subsequent studies, one in murine (15) and the other in human (46) B cells, several mutations in the BSAP binding site had no impact on the IL-4 inducibility of the ϵ germline promoter. However, the highly conserved C at position −5 in the BSAP binding region was spared by those mutational analyses. Notably, the activity of the BSAP-regulated promoters that contain the conserved C was consistently abolished or drastically reduced whenever that residue was mutated (19, 28, 47, 48). Indeed, the C at the border of the 3′ half-site of BSAP contributes critically to an optimal interaction with the N-terminal portion of the paired domain and thus to the overall affinity of the BSAP recognition sequence (40). Over and above other technical differences (e.g., boundaries of the reporter constructs, cell lines, or stimulation protocols), we believe that in the absence of the mutation at position −5, the ability of BSAP to interact with the germline promoter may not be significantly impaired.

Our present results strongly support a major role for BSAP in regulating the activity of the human ϵ germline promoter. Interestingly, the regulatory elements identified to date in this promoter are involved in either IL-4 (17) or CD40 (49) responsiveness. The BSAP site seems to be unique in that it contributes to both IL-4-dependent induction of ϵ germline transcription and CD40-mediated up-regulation of the promoter. This complexity of effect may reflect the ability of BSAP to act in concert with other nuclear factors to activate or repress target genes. Repression of the 3′α enhancer (hs1, 2) or activation of the 3′α enhancer-hs4 by BSAP involves the combined efforts of a common set of factors, such as octamer binding proteins, NF-κB-like complexes, and, in the case of the 3′α enhancer (hs1, 2), a novel G-rich DNA binding protein (47, 48). The choice between activation or repression seems to be strictly dependent on the topology of the binding sites for these factors and may result from direct occlusion of their activation domains or from the recruitment of another factor(s) that mediates repression or activation (50). This model is predicated on direct protein-protein interactions among multiple transcription factors. Indeed, BSAP was shown to directly interact with the POU domain of octamer binding proteins (47) and may well interact with other factors that bind the ϵ germline promoter.

All efforts to date to demonstrate signal-dependent post-translational modifications of BSAP, such as phosphorylation, have failed (43). This supports the possibility that BSAP, a constitutively expressed protein, controls activation of the ϵ germline promoter by interacting with other factors that are regulated by specific signals. In particular, an interaction between BSAP and NF-κB may be involved in the CD40-dependent up-regulation of ϵ germline promoter activity observed in our experiments. CD40

engagement has been shown to rapidly activate NF-κB and to support NF-κB-dependent gene expression in human B cells (51). Furthermore, ϵ germline transcripts were undetectable in B cells from p50/NF-κB −/− mice stimulated with IL-4, IL-5, and CD40 ligand (52), suggesting that NF-κB is essential for activation of transcription through the ϵ switch region. The human ϵ germline promoter contains two putative κB binding sites, one corresponding to the site identified in the IL-4-responsive element of the murine ϵ promoter (15), and the other located at position −43/−34, a few bases upstream of the BSAP binding site (L. De Monte, S. Monticelli, and D. Vercelli, manuscript in preparation). Reporter assays recently performed in our laboratory have shown that mutation of the NF-κB binding site at −43/−34 in the ϵ GL-luc construct resulted in a marked (>60%) inhibition of the CD40-dependent up-regulation of ϵ germline promoter activity. In contrast, IL-4 inducibility was unaffected (data non shown). It will be of interest to assess whether BSAP acts in concert with NF-κB and possibly with another factor(s) capable of binding the promoter.

A region that spans the 3′ portion of the BSAP binding site has been recently reported to contain a CD40-responsive element (49) distinct from the one that mediates IL-4 responsiveness of the ϵ germline promoter (17). By contrast, in our studies BSAP was shown to be essential for the response of the promoter to both IL-4 and CD40 engagement. Differences in a number of critical parameters may underlie this discrepancy, such as the sequence composition of the reporter vectors, the transfection approach, and, most importantly, the B cell lines used for reporter assays. Indeed, the vectors used in our study did not include the splice site for Cϵ1 and were transfected transiently, rather than stably, into EBV-negative B cell lines. The expression of EBV-encoded genes known to be functionally linked to the CD40 signaling pathway (36, 37, 53) may critically influence the outcome of reporter assays, possibly by inducing and/or up-regulating transcription factors that contribute to ϵ promoter regulation.

Although binding sites for BSAP have been identified in the vicinity of several germline promoters, such as murine γ1, γ2a, and α (45, 54), ϵ is the only promoter shown to be regulated by BSAP to date. If regulation of the ϵ germline promoter by BSAP really requires interaction(s) with other transcription factors, it is possible that binding sites for the appropriate BSAP partners are absent in promoters other than ϵ and/or are not accessible because of topologic constraints.

Except for BSAP, none of the nuclear factors reported to date to be critical for ϵ germline transcription (e.g., STAT6, C/EBP, NF-κB, or HMG-I(Y)) (15, 17, 42, 55) is expressed exclusively in B cells, yet germline transcription is exquisitely B cell-specific. We are currently investigating whether the B cell specificity of germline transcription is functionally linked to BSAP expression. An additional possibility is that BSAP contributes to coupling germline transcription and switching to cell proliferation. Indeed, switch recombination to γ1 and expression of γ1 and ϵ germline transcripts were severely reduced after incubation of activated murine B cells with DNA synthesis inhibitors (56). BSAP activity is known to be up-regulated by stimulation of murine B cells with mitogens or CD40 ligand, and down-regulated by cross-linking of OX40 ligand (39). Antisense-mediated down-regulation of BSAP resulted in a dramatic decrease in cell proliferation and in the inhibition of class switching to IgG1 induced by IL-4 and LPS (38). A clue to the mechanisms underlying the link between BSAP and B cell proliferation has been offered by recent results demonstrating that BSAP represses transcription of the tumor suppressor gene p53 (57). p53 appears to control the cell cycle by activating downstream target genes that arrest cells in late G1 phase and, furthermore, mediates apoptosis in a variety of cell types. BSAP expression in the early stages of B cell differentiation would inhibit p53 expression, leading to rapid growth and partial differentiation, while preventing cellular decisions concerning apoptosis.

In conclusion, among the several transcription factors that regulate ϵ germline expression identified to date, BSAP seems to be unique in that it is B cell-specific, is constitutively expressed rather than inducible, is involved in activation but not in repression of the promoter, and, most importantly, is at the intersection of two signaling pathways that are distinct but both critical for the induction of switching to IgE.

Acknowledgments

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IL-13 Down-Regulates CD14 Expression and TNF-α Secretion in Normal Human Monocytes[1]

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CD14, a glycosylphosphatidylinositol (GPI)-linked protein expressed on monocytes and neutrophils, regulates monocyte-lymphocyte interactions and serves as the LPS receptor. We showed previously that IL-4 down-regulates the expression of human CD14 by acting at the transcriptional level. We now investigate whether CD14 expression could also be regulated by IL-13, another member of the chromosome 5 cytokine gene family. IL-13 dose-dependently inhibited CD14 expression on human monocytes. By contrast, expression of CD23 and CD11b was enhanced strongly. Down-regulation of CD14 involved neither shedding nor activation of endogenous GPI anchor-cleaving enzymes. Indeed, soluble CD14 was not increased in the supernatants of IL-13-stimulated monocytes, and expression of CD55/DAF, another GPI-linked protein, was unaffected by IL-13. CD14 transcript levels were reduced sixfold in IL-13-treated monocytes. These results suggest that IL-13 down-regulates membrane CD14 by suppressing CD14 RNA expression. IL-13-dependent down-regulation of CD14 resulted in the inhibition of CD14-mediated events. Indeed, CD14-mediated release of TNF-α was inhibited markedly (~75%) in monocytes stimulated with LPS (100 ng/ml) after a 72-h preincubation with IL-13. However, IL-13 also directly inhibited monokine secretion, because it blocked PMA-induced, CD14-independent TNF-α release. Down-regulation of CD14 and TNF-α secretion may play a major role in the anti-inflammatory effects of IL-13 on LPS-stimulated monocytes. *The Journal of Immunology,* 1995, 155: 3145–3151.

CD14 is a glycosylphosphatidylinositol (GPI)[3]-linked glycoprotein expressed on monocytes, macrophages, and neutrophils (1, 2). CD14 has been shown to trigger monocyte activation (3) and to serve as the receptor for LPS (4). A soluble form of CD14, present in the serum at high concentrations (5), enables responses to LPS by $CD14^-$ cells, possibly by forming a complex with LPS and then binding additional membrane proteins (6, 7). In this capacity, CD14 plays a pivotal role in endotoxin-induced monokine release during Gram-negative infections (4, 8), as confirmed by in vivo studies with transgenic mice (9). Furthermore, CD14 plays an important role in the regulation of human T and B cell functions. Engagement of selected CD14 epitopes by specific mAbs terminates T cell proliferation by delivering a negative signal to T cells (10) and inhibits the synthesis of all Ig isotypes by acting at the B cell level (11).

CD14 expression is regulated by cytokines (reviewed in Ref. 12). We have shown that IL-4 down-regulates CD14 by acting at the transcriptional level (13). Recently, it has become clear that a number of functional properties of IL-4 are shared by IL-13 (14–16), a cytokine produced by T cells and mast cells that is also encoded in the cytokine family on chromosome 5 (14, 17–19). In particular, IL-13 has been shown to affect the morphology, phenotype, function, and cytokine production of human monocytes (14, 20–22). We investigated whether rIL-13 is able to regulate the expression of CD14 and CD14-dependent events in monocytes. We show herein that, similar to IL-4,

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[3] Abbreviations used in this paper: GPI, glycosylphosphatidylinositol; DAF, decay accelerating factor; MFI, mean fluorescence intensity; RT-PCR, reverse transcriptase-mediated PCR.

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Table I. *RT-PCR for monocytes genes: primers, product sizes, and annealing temperatures*

cDNA	Primer Pair (upper strand; lower strand)	Size (bp)	*T* (°C)
CD14	TAAAGGACTGCCAGCCAAGC AGCCAAGGCAGTTTGAGTCC	280	60
CD23	CGAAGTTCCTCCAGTTCCTGTGAAATCTGCGTGGACTG GAGGACAGACACAGGCTCCAAACTCCACTAACCAGAG	402	65
CD55	GCTGCTGCTGGTGCTGTTGTG GCCACCTGGTACATCAATCTGAC	475	65
β-actin	GGATCTTCATGAGGTAGTCAGTCAGG GAGCACAGAGCCTCGCCTTTGC	632	65

IL-13 down-regulates CD14 expression. CD14 down-regulation resulted neither from increased shedding of soluble CD14 nor from triggering of endogenous GPI anchor-cleaving enzymes but, rather, was due to inhibition of CD14 RNA expression.

Materials and Methods

Reagents

Purified and phycoerythrin-conjugated Leu-M3 mAb (anti-CD14, clone P9, IgG2b), isotype control mAbs, and FITC-conjugated goat anti-mouse Ig (GAMIG) were purchased from Becton Dickinson (Mountain View, CA). IOM2 mAb (anti-CD14, IgG2a) and IOB8 mAb (anti-CD23, IgG1) were purchased from AMAC (Westbrook, ME). OKM1 mAb (anti-CD11b, IgG2b) was obtained from Ortho Diagnostics (Raritan, NJ). MEM-18 mAb (anti-CD14, IgG1) (5) and 1A10 mAb (anti-CD55/decay accelerating factor, DAF: IgG2a) (23) were a kind gift of Dr. V. Bazil Systemix, Palo Alto, CA, and Dr. E. Medof (Case Western Reserve University, Cleveland, OH), respectively. An IgG2b isotype control was purchased from Zymed (South San Francisco, CA). *Escherichia coli*-derived, purified human IL-13 (specific activity 1.6×10^6 U/mg) was obtained as described previously (14) and was kindly provided by Dr. R. de Waal Malefyt (DNAX Research Institute of Cellular and Molecular Biology, Palo Alto, CA), whereas purified human rIL-12 (specific activity 1.7×10^8 U/mg) was a kind gift of Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ). Purified human rIL-4 (specific activity 2×10^6 U/mg) was purchased from R&D Systems (Minneapolis, MN). LPS from *Saccharomyces minnesota* was obtained from Sigma Chemical Co. (St. Louis, MO). TNF-α concentrations in culture supernatants were determined by ELISA (Endogen, Boston, MA; lower limit of sensitivity 10 pg/ml). The human neuroblastoma cell line IMR-32 was obtained from American Type Culture Collection (Rockville, MD).

Isolation and culture of PBM

Monocytes were prepared as described previously (24). In brief, PBMC were isolated from heparinized venous blood of healthy human donors by Ficoll-Hypaque density gradient centrifugation. The cells were then resuspended in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% AB^+ serum (Sigma), 2 mM glutamine, 50 μg/ml streptomycin, and 100 U/ml penicillin (complete medium), and adhered for 1 to 4 h at 37°C in a 5% CO_2 atmosphere in plastic Petri dishes, each containing 10 to 15×10^7 mononuclear cells. After removing the nonadherent cells, the dishes were washed extensively with warm medium and then incubated with cold PBS on ice for 15 min. Adherent monocytes were recovered subsequently by vigorous pipetting, washed, and resuspended in complete medium at 2×10^6 cells/ml. Cell viability, as determined by trypan blue exclusion, was always >95%. Purified monocytes (2×10^6 cells/ml) were cultured in the presence or absence of cytokines. Preliminary experiments established that optimal down-regulation of CD14 was observed using IL-13 at 100 U/ml. This concentration, therefore, was used in all subsequent experiments. Supernatants for TNF-α assessment were collected after 20 h and stored at −20°C.

Immunofluorescence staining

For direct immunofluorescence, purified monocytes (2×10^6 cells/ml) in staining buffer (RPMI 1640–10% AB^+ serum, containing 0.01% sodium azide) were incubated with fluorochrome-conjugated mAbs or isotype controls for 40 min at 4°C. The cells were then washed extensively and fixed in 2% *p*-formaldehyde. For indirect immunofluorescence, monocytes were incubated with purified Abs followed by FITC-conjugated GAMIG. Percentages of positive cells and mean fluorescence intensity (MFI) were analyzed by a FACScan (Becton Dickinson) gating on the monocyte population, as defined by forward and side light scatter.

Biosynthetic labeling

Purified monocytes (2×10^6 cells/ml) were incubated in the presence of medium or rIL-13 (100 U/ml) for 42 h. Cells were then washed and incubated for 6 h in methionine- and cysteine-free RPMI 1640, supplemented with 10% dialyzed AB^+ serum and containing [^{35}S]methionine and [^{35}S]cysteine (Pro-mix, Amersham, Arlington Heights, IL; 10 mCi/ml) in the presence or absence of rIL-13. The monocyte supernatants (i.e., the labeling medium) were then collected and centrifuged for 1 h at $100{,}000 \times g$. For immunoprecipitation, supernatants were precleared by overnight incubation with normal mouse serum (2 μl) and 20 μl of packed protein G-agarose beads (Boehringer Mannheim, Indianapolis, IN). The following day, supernatants were recovered and incubated for 4 h with anti-CD14 mAb P9 or isotype control (10 μg) and protein G-agarose beads (20 μl). The beads were then washed extensively in lysis buffer, resuspended in loading buffer, and boiled. Samples containing equal amounts of protein were analyzed by 12% SDS-PAGE under reducing conditions followed by autoradiography.

Semiquantitative reverse transcriptase-mediated PCR

Levels of mRNA for CD14 and other monocyte surface molecules were assessed by a semiquantitative reverse transcriptase-mediated PCR (RT-PCR) assay. Total cellular RNA was extracted by the method of Chomczynski (25). cDNA was synthesized using Moloney leukemia virus reverse transcriptase (Life Technologies) and random primers. PCR primers were 5′-labeled using T4 polynucleotide kinase (Life Technologies; 5 U/10 pmol of primer) and 30 μCi [γ-^{32}P]ATP (3000 Ci/mmol, Amersham). For each reaction, one primer was labeled and added in a 1:10 molar ratio with cold primer. The number of amplification cycles chosen for each primer pair was such that the maximum signal intensity for a set of samples was within the linear portion of a product vs template amplification curve. Each sample was assayed initially for amplifiable cDNA using primers specific for β-actin, which is known to be unaffected by our experimental conditions. Based on the amount of β-actin PCR product in this assay, aliquots of RT product representing equivalent amounts of β-actin were amplified using primers for CD14, CD23, and CD55 (Table I). Each reaction was performed in a final volume of 20 μl, containing 20 pmol of each primer, cDNA (2 μl), PCR reaction buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.1 μl of Tween 20, and 0.25 U of Taq polymerase (Promega). Amplifications were performed in a thermocycler (Omnigene, Hybaid, Teddington, UK) as follows: 1 min at 94°C, followed by 20 to 35 amplification cycles (primer annealing temperature as specified in Table I, 1 min), 1 min at

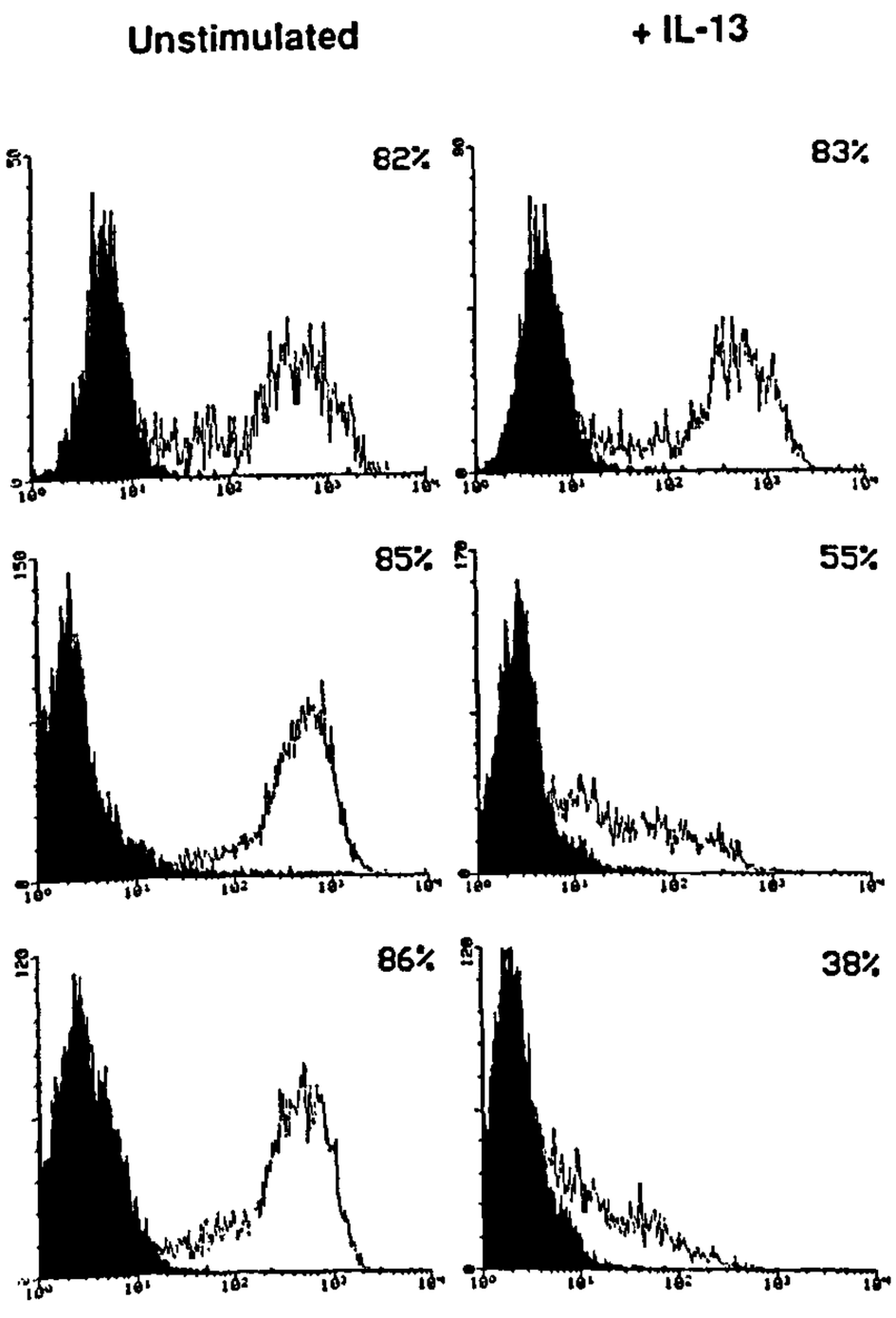

FIGURE 1. Kinetics of IL-13-dependent inhibition of CD14 expression. Human monocytes (2×10^6 cells/ml) were cultured for 24, 48, and 72 h in the presence or absence of IL-13 (100 U/ml). CD14 expression was assessed by indirect immunofluorescence, using mAb Leu-M3 (open profile) or an isotype-matched control (solid profile). The data are representative of five separate experiments.

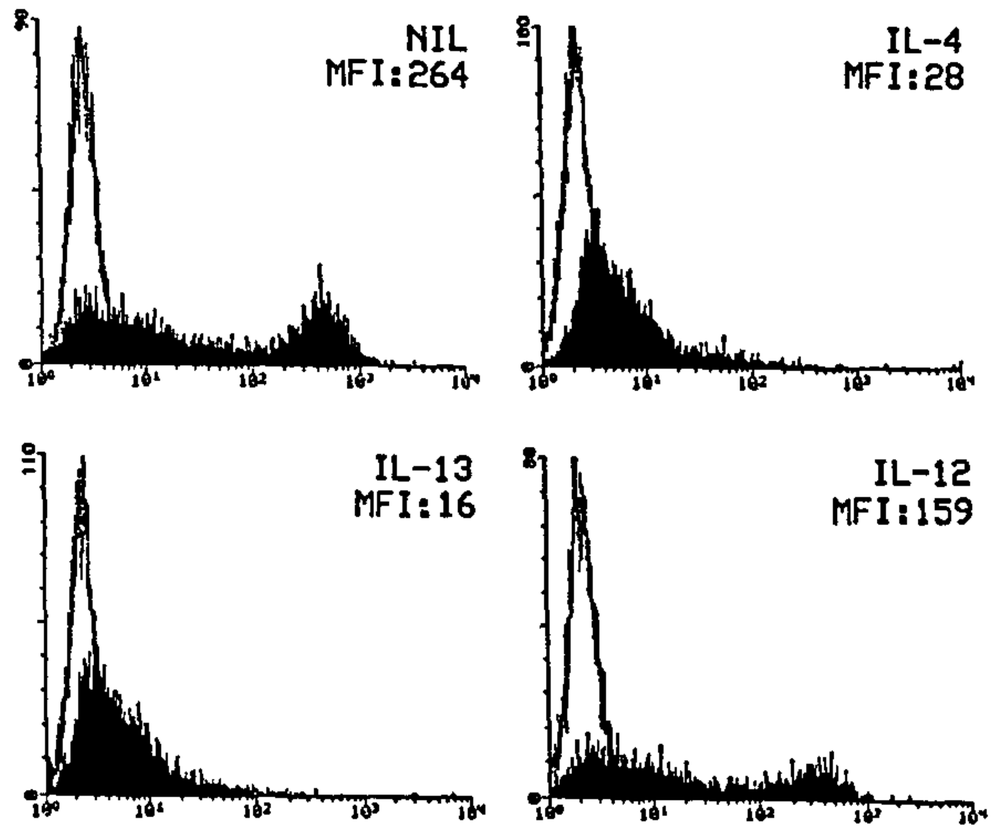

FIGURE 2. Cytokine-dependent modulation of CD14 expression. Human monocytes (2×10^6 cells/ml) were cultured for 48 h in the presence of medium, IL-4 (5 ng/ml), IL-13 (100 U/ml), and IL-12 (1 ng/ml). CD14 expression was then assessed by indirect immunofluorescence, using mAb Leu-M3 (solid profile) or an isotype control (open profile). The data are representative of four separate experiments.

74°C, and maintenance at 4°C until analysis. Aliquots (10 μl) of each amplification were analyzed by electrophoresis on 4% acrylamide gels followed by autoradiography using Kodak diagnostic film X-OMAT AR. Scanning densitometry analysis was performed on a 300E Computer Densitometer (Molecular Dynamics, Sunnyvale, CA). DNA sizes were compared against DNA m.w. standards derived from an *Hpa*II digest of pBluescript II SK and KS (Stratagene, La Jolla, CA), labeled with [α-^{32}P]dCTP (Amersham) using Klenow DNA polymerase I (Promega).

Results

rIL-13 down-regulates CD14 expression on normal human monocytes

To assess whether the cytokine IL-13 plays a role in the regulation of CD14 expression, normal human monocytes were incubated with rIL-13 (100 U/ml) and CD14 expression was assessed by flow cytometry at various time points. Figure 1 shows that CD14 expression remained virtually stable in unstimulated monocytes. By contrast, the percentage of CD14$^+$ cells progressively decreased in the presence of IL-13 and was reduced by >50% at day 3. Figure 2 shows that, as expected (3), CD14 down-regulation was also observed in monocytes stimulated with IL-4, whereas an unrelated cytokine, rIL-12, only modestly decreased CD14 MFI and had no effect on the percentage of CD14$^+$ cells (data not shown). Finally, the down-regulating effect of IL-13 was CD14-specific, because incubation with IL-13 substantially enhanced the expression of CD11b (Fig. 3, *top*) and CD23 (Fig. 3, *bottom*). Thus, IL-13 can exert specific and opposite effects on the expression of different monocytic Ags.

IL-13-induced down-regulation of CD14 involves neither shedding nor GPI-anchor cleavage

CD14 is anchored to the cell membrane by a GPI linkage (1) and is shed upon activation of monocytes (26). Indeed, shedding was shown to be responsible for down-regulation of CD14 by phorbol esters and IFN-γ (26). We investigated, therefore, whether the IL-13-induced decrease in membrane CD14 resulted from shedding. To this purpose, monocytes were cultured for 42 h in the presence or absence of rIL-13 (100 U/ml) and then labeled for 6 h with [^{35}S]methionine/cysteine. Supernatants were then collected, and immunoprecipitation was performed with anti-CD14 mAb P9. Figure 4 shows that a band corresponding to soluble CD14 was precipitated from supernatants of unstimulated monocytes by mAb P9 (*lane 1*), but not by an IgG2b isotype control (*lane 3*). Soluble CD14 did not increase after incubation with rIL-13 (*lane 2*). These results ruled out protein shedding as the mechanism responsible for IL-13-induced down-regulation of membrane CD14.

To test whether triggering of endogenous GPI anchor-cleaving enzyme(s) was responsible for IL-13-dependent

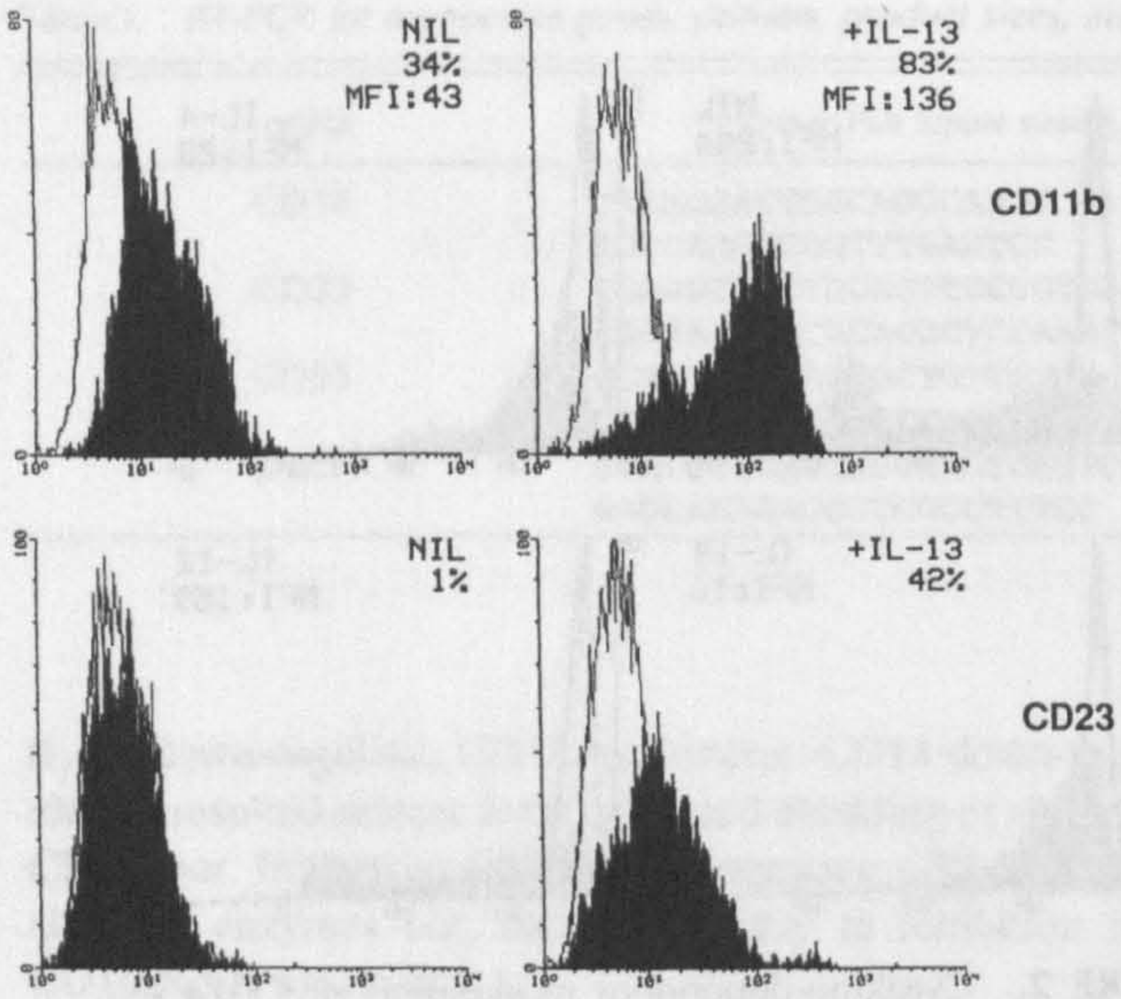

FIGURE 3. IL-13 up-regulates CD11b and induces CD23 on human monocytes. Human monocytes (2×10^6 cells/ml) were cultured for 48 h in the presence or absence of IL-13 (100 U/ml). Expression of CD11b and CD23 was assessed by indirect immunofluorescence, using mAb OKM1 or IOB8 (solid profile) or isotype controls (open profile). The data are representative of three separate experiments.

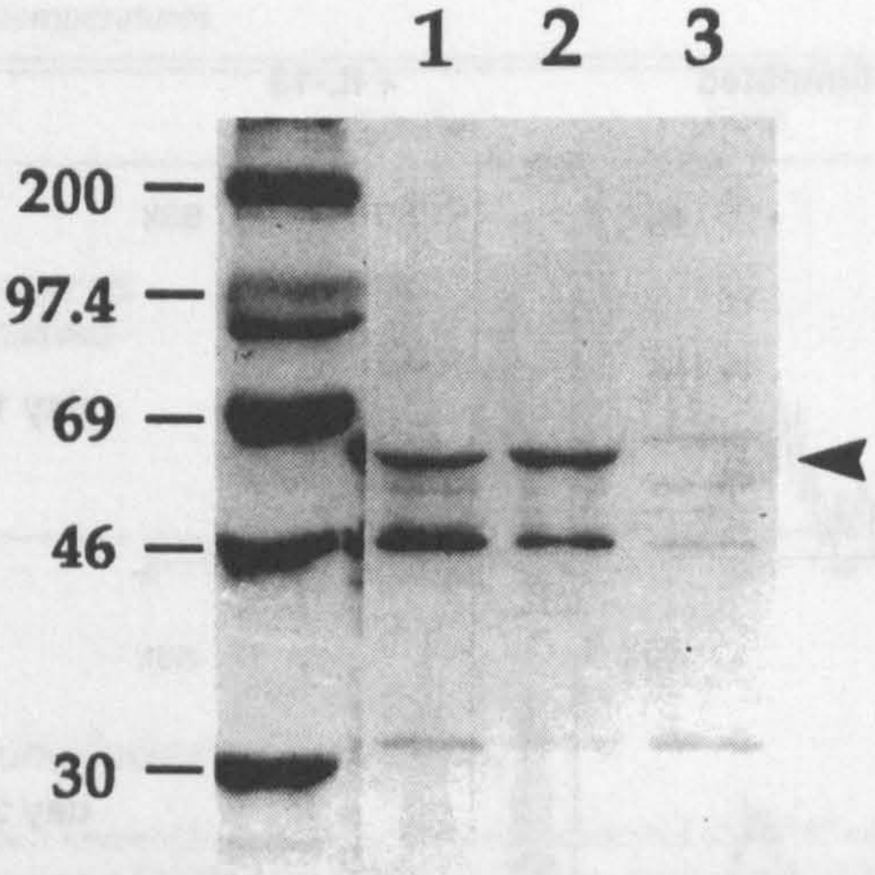

FIGURE 4. CD14 shedding is not affected by IL-13. Human monocytes (2×10^6 cells/ml) were cultured for 42 h in the absence or presence of rIL-13 (100 U/ml) and labeled with [^{35}S]methionine/cysteine during the last 6 h of culture. Cell supernatants were then collected and immunoprecipitated with anti-CD14 mAb P9 or isotype control (10 μg) and analyzed by 12% SDS-PAGE under reducing conditions, followed by autoradiography. *Lane 1*, supernatants from unstimulated monocytes, immunoprecipitated with mAb P9; *lane 2*, supernatants from IL-13-stimulated monocytes, immunoprecipitated with mAb P9; *lane 3*, supernatants from unstimulated monocytes, immunoprecipitated with IgG2b isotype control. The data are representative of two separate experiments.

down-modulation of CD14, monocytes incubated with IL-13 for 24 and 48 h were assessed by immunofluorescence for the expression of CD55 (DAF), a GPI-linked protein (27, 28) expressed at high levels on unstimulated monocytes (23). Figure 5 shows that IL-13 had a modest effect on CD55/DAF expression, indeed not comparable with the one observed on CD14. Thus, IL-13-induced down-regulation of CD14 seems to involve neither cleavage of GPI anchors nor shedding.

IL-13 down-regulates CD14 transcript levels

To elucidate further the mechanism(s) of CD14 down-modulation by IL-13, we then investigated the effect of IL-13 on CD14 transcript levels using a semiquantitative RT-PCR assay. Human monocytes were cultured for 36 h in the absence or presence of rIL-13 (100 U/ml), total RNA was extracted, and cDNA was synthesized and amplified with radioactive primers after normalizing for amplifiable cDNA levels. Figure 6 shows that CD14 RNA was well expressed in monocytes cultured in medium but was down-regulated markedly (sixfold) after incubation with rIL-13. Consistent with the Ag expression patterns observed by immunofluorescence, IL-13 induced a modest (1.8-fold) decrease in CD55 RNA and a marked (threefold) up-regulation of CD23. The RT-PCR was specific, because no band corresponding to CD14, CD23, or CD55 RNA was amplified from cDNA of the neuroblastoma cell line IMR-32. These results suggest that IL-13-induced down-regulation of membrane CD14 results from a reduction in transcript levels.

IL-13 inhibits both CD14-mediated and -independent secretion of TNF-α

CD14 has been shown to act as the LPS receptor (4), thus mediating LPS-induced monokine release (8). We investigated, therefore, whether IL-13-dependent down-regulation of CD14 could affect LPS-induced release of TNF-α. Table II shows that purified monocytes secreted large amounts of TNF-α in response to a 20-h stimulation with LPS (100 ng/ml). TNF-α release was CD14-dependent, because it was virtually abolished by mAb MEM-18, which recognizes a CD14 epitope in the LPS-binding site (8, 29). A 72-h preincubation with IL-13 strongly (~75%) inhibited LPS-induced TNF-α release. MEM-18 mAb completely abrogated the residual response to LPS of IL-13-stimulated monocytes, suggesting that the latter was mediated by residual CD14 expression. Notably, Table II also shows that preincubation with IL-13 markedly (~76%) inhibited the CD14-independent secretion of TNF-α induced by PMA (8). Thus, IL-13 inhibits both CD14-mediated and -independent TNF-α release.

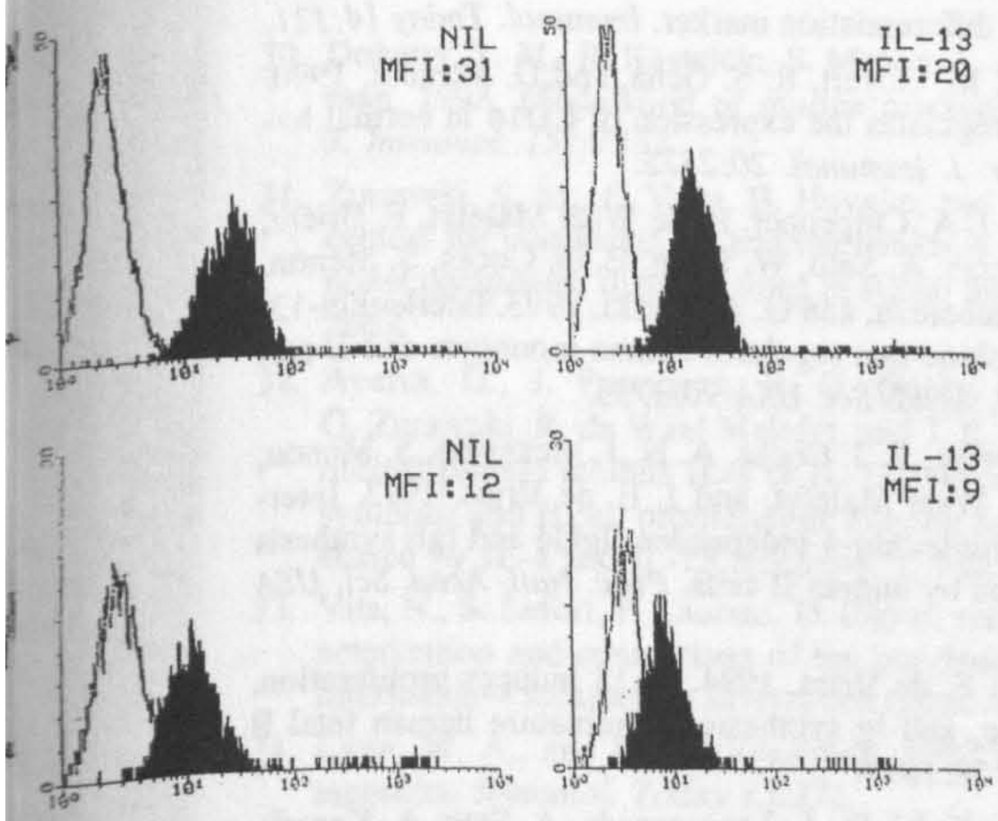

FIGURE 5. Effect of IL-13 on CD55 expression. Human monocytes (2×10^6 cells/ml) were cultured for 24 and 48 h in the presence or absence of IL-13 (100 U/ml). CD55 expression was assessed by indirect immunofluorescence, using mAb 1A10 (solid profile) or an isotype control (open profile). The data are representative of three separate experiments.

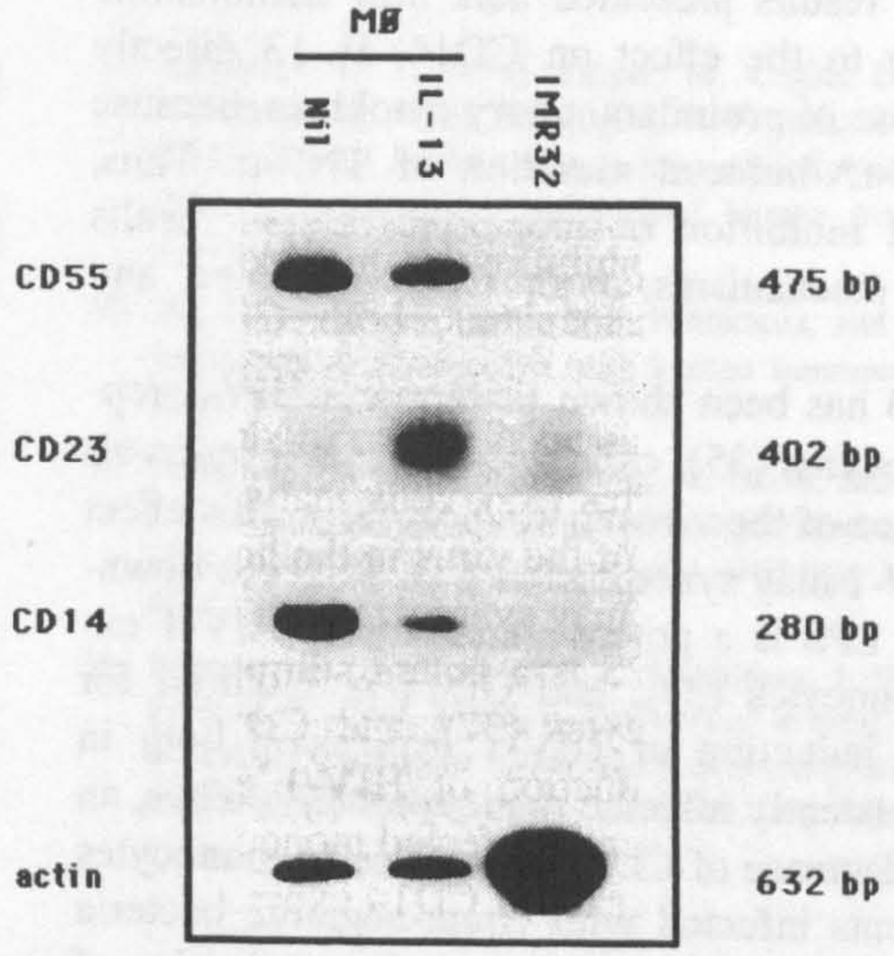

FIGURE 6. IL-13 down-regulates CD14 transcript levels. Total RNA was isolated from peripheral blood monocytes cultured for 36 h in the absence or presence of rIL-13 (100 U/ml), cDNA was synthesized, and analyzed by semiquantitative RT-PCR. Amplification cycles used were as follows: 35 for CD55 and CD23, 20 for CD14, and 24 for β-actin.

Discussion

The cytokine IL-13 has been shown to exert pleiotropic effects on monocytes in both mice (30) and humans (20). IL-13-dependent down-modulation of human CD14 had been observed previously (20); however, the mechanisms underlying this effect of IL-13 were not investigated. CD14 expression can be regulated through different pathways. IFN-γ, phorbol esters, and calcium ionophore down-modulate CD14 by inducing activation of membrane-bound protease(s) with rapid shedding (26). IL-4, on the other hand, reduces CD14 by inhibiting the expression of CD14 RNA (13). Our present data show that IL-13 resembles IL-4 in its ability to interfere with CD14 RNA expression. Although the sequence homology between IL-13 and IL-4 is only ~30%, all residues that contribute to the hydrophobic core of IL-4 are conserved or have conservative hydrophobic replacements in IL-13 (31). Furthermore, receptors for IL-4 and IL-13 may share a subunit (31). Indeed, an IL-4 mutant protein that binds IL-4R with high affinity competitively inhibits receptor binding of both IL-4 and IL-13 and blocks induction of IgG4 and IgE synthesis by both cytokines (32). However, IL-4 and IL-13 as well as their receptors are by no means identical. IL-13 does not bind to COS-3 cells transfected either with cDNAs for the 130-kDa IL-4R and/or the γ-chain (33). Furthermore, unlike IL-4, IL-13 has no effects on human T cells (31) and does not seem to activate directly human pre-B cells (16). The elucidation of the respective roles of IL-4 and IL-13 in physiologic conditions will require information about the structure and distribution of IL-13 receptors, as well as a detailed analysis of the mechanisms that control the expression of these cytokines.

Table II. *Preincubation with IL-13 inhibits both CD14-mediated and V-independent release of TNF-α from human monocytes*[a]

Stimulant	IL-13	Anti-CD14 mAb	TNF-α (pg/ml)
Nil	−	−	47 ± 7
	+	−	15 ± 4
LPS	−	−	25,225 ± 5,782
	−	+	154 ± 15
	+	−	7,575 ± 2,832
	+	+	61 ± 10
PMA	−	−	2,775 ± 1,062
	+	−	675 ± 170

[a] Human monocytes (2×10^6 cells/ml) were cultured for 72 h in the presence or absence of IL-13 (100 U/ml), washed, resuspended in complete medium, and stimulated with LPS (100 ng/ml) or PMA (50 ng/ml) in the presence or absence of anti-CD14 mAb MEM-18 (1:200). After 20 h, supernatants were harvested and TNF-α concentrations were determined by ELISA. The table shows the mean ± SE of results obtained in four experiments.

The ability of IL-13 to down-regulate the expression of CD14 has important functional implications. We and others have shown that the LPS-dependent release of proinflammatory monokines is decreased strikingly in IL-13-treated monocytes, whereas IL-1ra secretion is enhanced (20). Although these results pointed to a major anti-inflammatory effect of IL-13, the mechanisms underlying such an effect remained to be determined. Because LPS is the stimulus commonly used to elicit the release of proinflammatory monokines and CD14 is the major LPS receptor (reviewed in Ref. 34), the anti-inflammatory effect of IL-13 in LPS-driven systems may depend entirely on CD14 down-modulation. Indeed, we (Table II) and others (8) have shown that addition of Abs specific for the LPS-binding site on CD14 is sufficient to block LPS-induced monokine release by untreated (i.e., CD14-bearing)

monocytes. The results presented here now demonstrate that, in addition to the effect on CD14, IL-13 directly inhibits the release of proinflammatory cytokines, because it suppresses PMA-induced secretion of TNF-α. Thus, IL-13-dependent inhibition of monokine release results from complex mechanisms, both CD14-mediated and -independent.

Finally, IL-13 has been shown to suppress HIV-1 replication in monocytes (35), cells that serve as a reservoir for the persistence of the virus in the host (36). This effect of IL-13 on HIV-1 may synergize with its ability to down-regulate CD14. LPS is a potent stimulator of HIV-1 expression in monocytes (37), and CD14 is required for LPS-dependent induction of HIV-1 expression both in chronically and latently infected monocytes (38). Thus, an IL-13-induced decrease of CD14 expression in monocytes from HIV patients infected with Gram-negative bacteria would raise the threshold for LPS-dependent activation of HIV-1 expression, thus contributing to maintaining proviral latency.

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Immunology

Deletional switch recombination occurs in interleukin-4-induced isotype switching to IgE expression by human B cells

(immunoglobulin/DNA rearrangement)

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ABSTRACT **There is controversy as to whether deletional rearrangement occurs between the IgM and IgE switch regions (S_μ and S_ε, respectively) during switching to the IgE isotype. We have addressed the issue by stimulating normal human B cells, sorted for lack of expression of surface IgE, to produce IgE by infection with Epstein–Barr virus (EBV) in the presence of interleukin 4 (IL-4). Genomic DNA was amplified for S_μ/S_ε switch junction fragments by utilizing the nested-primer polymerase chain reaction. Switch junction fragments were amplified from B cells infected with EBV in the presence of IL-4 but not from B cells infected with EBV alone. The DNA sequence of these "switch fragments" revealed direct joining of S_μ to S_ε in each case. The recombination sites within S_μ were clustered within 900 base pairs at the 5′ end of the switch region, suggesting that there are "hot spots" for recombination within S_μ. The S_ε recombination sites were scattered throughout the S_ε region. These findings indicate that IL-4-induced isotype switching to IgE production in human B cells is accompanied by DNA rearrangements with joining of S_μ to S_ε.**

Diversification of antibody biological function is achieved by the process of heavy chain class switching. Isotype switching occurs by molecular recombination between switch (S) regions (1–3) located upstream of each heavy chain constant (C_H) region gene with intramolecular deletion of the DNA segment between the recombined S regions (4–6). Mitogens and T-cell-derived lymphokines regulate isotype switching. These agents presumably modulate the accessibility of specific S regions to a putative switch recombinase (7–9).

Switching to IgE requires interleukin 4 (IL-4) and a B-cell activation signal provided by T cells (10, 11) or by infection with Epstein–Barr virus (EBV) (12, 13). Recent studies have suggested that, unlike switching to other isotypes, switching to IgE expression may not involve DNA rearrangement (14–16) but would instead occur by alternative splicing of a long mRNA transcript or transsplicing of a productive variable–diversity-joining $(VDJ)_\mu$ transcript to a downstream germ-line transcript. On the other hand, Thyphronitis *et al.* (17) have recently demonstrated by Southern blot analysis the rearrangement of one $C_{H\varepsilon}$ gene and deletion of both $C_{H\mu}$ genes in two EBV-transformed IgE-secreting B-cell clones, suggesting that deletional mechanisms could underlie isotype switching to IgE. However, because these two clones were derived from an unfractionated population of B cells, it was not clear whether they represented cells that were actually induced by IL-4 to undergo isotype switching. More importantly, because of its limited sensitivity, Southern blot analysis may not be useful for the study of populations of B cells in which only a small percentage have undergone switching.

Here we utilized the nested-primer polymerase chain reaction (PCR) to demonstrate that deletional recombination occurs in human B cells, sorted for lack of expression of surface IgE ($sIgE^-$) and stimulated to switch to IgE synthesis by EBV infection in the presence of recombinant IL-4 (rIL-4). Sequence analysis of amplified S_μ/S_ε "switch fragments" revealed direct joining of S_μ to S_ε with a clustering of S_μ recombination sites within the 5′ end of the S region, suggesting possible "hot spots" for recombination within S_μ. Thus, nested-primer PCR provided the sensitivity to analyze switching in polyclonal populations of cells that was not available with Southern blotting techniques.

MATERIALS AND METHODS

B-Cell Preparation/EBV Transformation. B-cell-enriched populations were prepared from peripheral blood mononuclear cells of nonatopic subjects (serum IgE < 70 international units/ml) as previously described (10). $sIgE^-$ B cells were sorted as previously described (13). EBV B-cell transformation in the presence or absence of rIL-4 has been described (18). Growth and passaging of the cells has been previously described (13).

IgE Assay. Cell lines were cultured at 0.5×10^6 cells per ml and supernatants were collected for assay after 3–4 days of growth. IgE was measured by a radioimmunoassay (19) with a limit of sensitivity of 150 pg/ml.

Preparation of Cellular DNA and Southern Blotting. High molecular weight DNA was prepared from 50×10^6 cultured cells by utilizing the A.S.A.P. genomic DNA isolation kit (Boehringer Mannheim). After cleavage with restriction endonucleases, the DNA was fractionated in 0.7% agarose gels and transferred to nylon membranes (Schleicher and Schuell). The $C_{H\varepsilon}$ probe was prepared by nick-translation (ref. 20, pp. 10.6–10.10) of the gel-purified 0.88-kilobase (kb) *Hinf*I fragment encompassing the first two exons of the $C_{H\varepsilon}$ gene and the introns bordering $C_{H\varepsilon 2}$ (described in ref. 13). Blots were hybridized and washed as described in the protocol for Nytran membranes (Schleicher and Schuell).

PCR Primers, Amplification of S_μ/S_ε Switch Fragments. Thirty-base-pair (bp) single-stranded DNA oligonucleotide primers for PCR were prepared by utilizing the PCR-Mate DNA synthesizer (Applied Biosystems). PCR amplification of the S_μ/S_ε fragment from the IgE-secreting plasmacytoma U266 (21) was performed with primers S6/S4 (see Fig. 2) in a reaction mixture containing each dNTP at 200 μM, 5 mM KCl, 1.0 mM $MgCl_2$, 10 mM Tris·HCl (pH 8.3), 0.01% gelatin, 1 mM dithiothreitol, 150 ng of each PCR primer, 1 μg of genomic template DNA, 2.5 units of AmpliTaq DNA poly-

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Abbreviations: S, switch; C_H, heavy chain constant; VDJ, variable–diversity–joining; EBV, Epstein–Barr virus; IL-4, interleukin 4; rIL-4, recombinant IL-4; $sIgE^-$, surface IgE-negative.

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merase (Perkin–Elmer), and 0.5 unit of Perfect Match polymerase enhancer (Stratagene) in a 50-μl volume under a 50-μl layer of mineral oil. The reaction mixture was cycled in a Perkin–Elmer/Cetus DNA thermal cycler for 35 cycles with 1 min at 94°C, 1 min at 65°C, and 4.5 min at 72°C. PCR amplification of S_μ/S_ε fragments from the EBV-transformed B cell DNA was similarly performed with primers S6/S4 followed by a second round of PCR utilizing the nested primers S8/S3 (Fig. 2) in a reaction mixture containing the same concentrations of all of the above constituents, except for 1.5 mM $MgCl_2$ and a 5-μl aliquot of the previous PCR reaction mixture that served as DNA template. The reaction mixture was cycled in the thermal cycler under the same conditions as described above.

Purification, Cloning, and Sequencing of PCR-Amplified DNA Fragments. PCR-amplified fragments were fractionated on 5% polyacrylamide gels, electroeluted from cut sections of the gel, and isolated by precipitation with ethanol (ref. 20, pp. 6.28–6.29, 6.39–6.44). Purified fragments were ligated into the *Sma* I site of pUC19 (22). Regions of the cloned fragment were subcloned in the replicative form I DNA for M13mp18 and M13mp19 (Pharmacia LKB), and single-stranded template DNA was isolated according to the protocols of New England BioLabs. All cloning steps utilized the *Escherichia coli* strain JM109 (23), a recombination-deficient strain that protects against bacterial-directed mutation or recombination within cloned fragments. Constructs were sequenced by the dideoxy chain-termination method (24) using Sequenase Version 2.0 (United States Biochemical). Additional 17-bp sequencing primers were prepared with the PCR-Mate DNA synthesizer.

RESULTS

Analysis of Cell Lines. Table 1 lists the cell lines studied. IgE was detected only in culture supernatants of B cells infected with EBV and cultured in the presence of IL-4. The percentage of cells in each cell line with intracytoplasmic IgE never exceeded 5% (13), even in the A5 cell line, which secreted IgE in levels comparable to those secreted by the U266 plasmacytoma line.

Table 1. Cell lines

Line	Sorted*	IL-4†	Time in culture, days	IgE, pg/ml
U266	NA	NA	NA	74,909
A5	−	+	>180	59,797
Semie	−	−	>180	<150
HJS6	+	+	43	2,199
HJS7	+	+	44	5,687
HJS02	+	−	57	<150

NA, not applicable.
*A + denotes that B cells were sorted for lack of expression of surface IgE prior to initiation of culture.
†Cell lines were incubated with (+) or without (−) rIL-4 at the time of EBV infection. rIL-4 was maintained in the media of the + cell lines.

Southern Blot Analysis of IgE-Secreting B-Cell Lines. We performed Southern blot analysis (Fig. 1*A*) on the high-IgE-producing line A5 and on the non-IgE-producing line Semie, which were derived from unsorted B cells from two different donors. A5 had been cultured with rIL-4, while Semie had been grown in the absence of rIL-4. U266 and a λ library clone derived from human placenta that contains the S_ε and $C_{H\varepsilon}$ region in the unrearranged configuration (25) were used as controls. The ε λ clone shows no rearrangement involving S_ε (5.5-kb *Pst* I fragment). U266 DNA had a single rearrangement (3.3-kb *Pst* I fragment) corresponding to the S_μ/S_ε recombination event depicted in Fig. 1*B*. The single rearrangement in U266 indicates either that both chromosomes are identically rearranged or that only one copy of this region is present in the cell line. The second explanation is more likely, since karyotype analysis revealed that U266 is not diploid for chromosome 14 (data not shown). The Southern blot pattern for both the IgE-secreting and non-IgE-secreting cell lines was that of an unrearranged chromosome (5.5-kb *Pst* I fragment). These results indicated that Southern blot analysis for the detection of S_μ/S_ε recombination events in polyclonal populations of B cells is not informative.

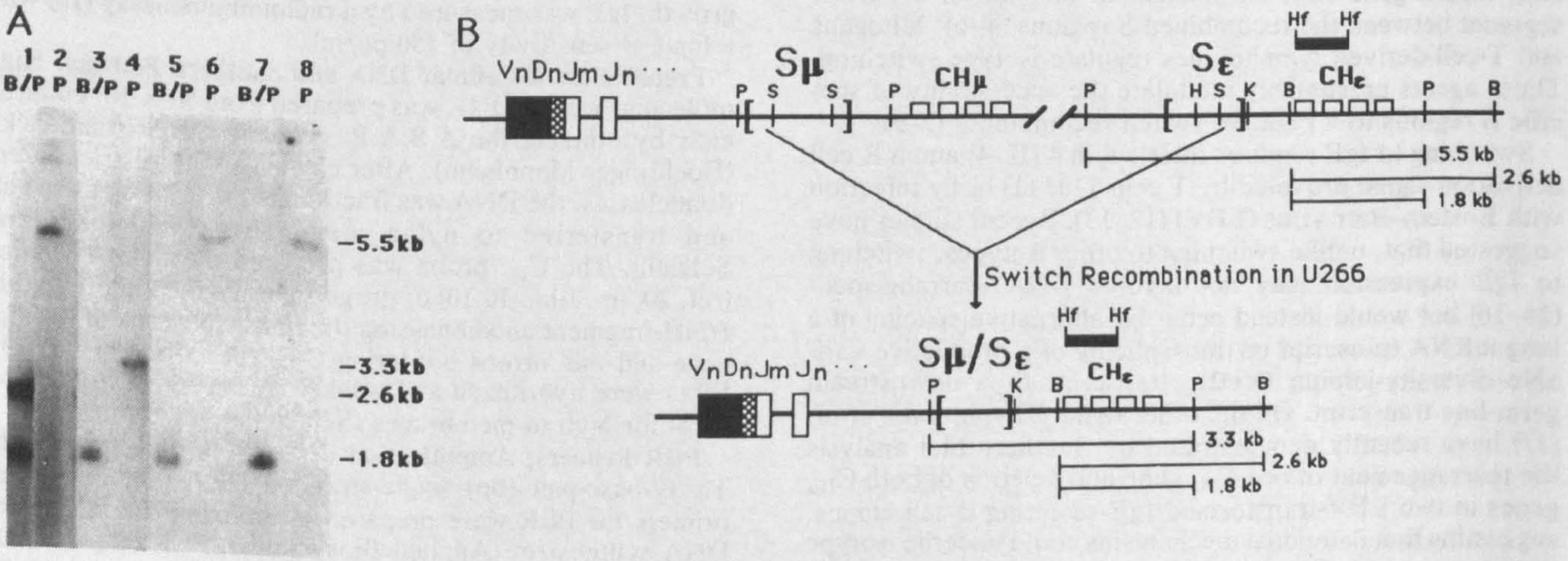

FIG. 1. Arrangement of the S_ε region in U266, IgE-secreting (A5), and non-IgE-secreting (Semie) lymphoblastoid lines. (*A*) Southern blot analysis of cell lines compared with the λ clone 38A (25), isolated from a human placenta genomic library. High molecular weight DNA samples isolated from the ε λ clone (lanes 1 and 2), U266 (lanes 3 and 4), and lymphoblastoid cell lines (A5 in lanes 5 and 6 and Semie in lanes 7 and 8) were digested with *Bam*HI/*Pst* I (B/P) or with *Pst* I (P) alone. Samples on this Southern blot were hybridized with the 0.88-kb *Hin*fI fragment encompassing $C_{H\varepsilon 1}$ and $C_{H\varepsilon 2}$ (shown above $C_{H\varepsilon}$ in *B*). All four DNA samples show the 1.8-kb *Bam*HI–*Pst* I fragment encompassing $C_{H\varepsilon}$ that does not undergo rearrangement (lanes 1, 3, 5, and 7). The λ clone has an unrearranged S_ε region represented by a 5.5-kb *Pst* I fragment (lane 2). The additional bands in lanes 1 and 2 are the result of incomplete digestion with *Pst* I. U266 DNA has only a 3.3-kb *Pst* I fragment, consistent with rearrangement involving the S_ε region (lane 4). Both A5 and Semie cellular DNA have a 5.5-kb *Pst* I fragment, denoting an unrearranged S_ε region (lanes 6 and 8). No other bands were detected in the lymphoblastoid cell lines, even with overexposure of the autoradiogram. (*B*) Diagrammatic summary of the rearrangement event that occurred in U266. Brackets represent the probable extent of the switch regions. B, *Bam*HI; H, *Hin*dIII; Hf, *Hin*fI; K, *Kpn* I; P, *Pst* I; S, *Sac* I.

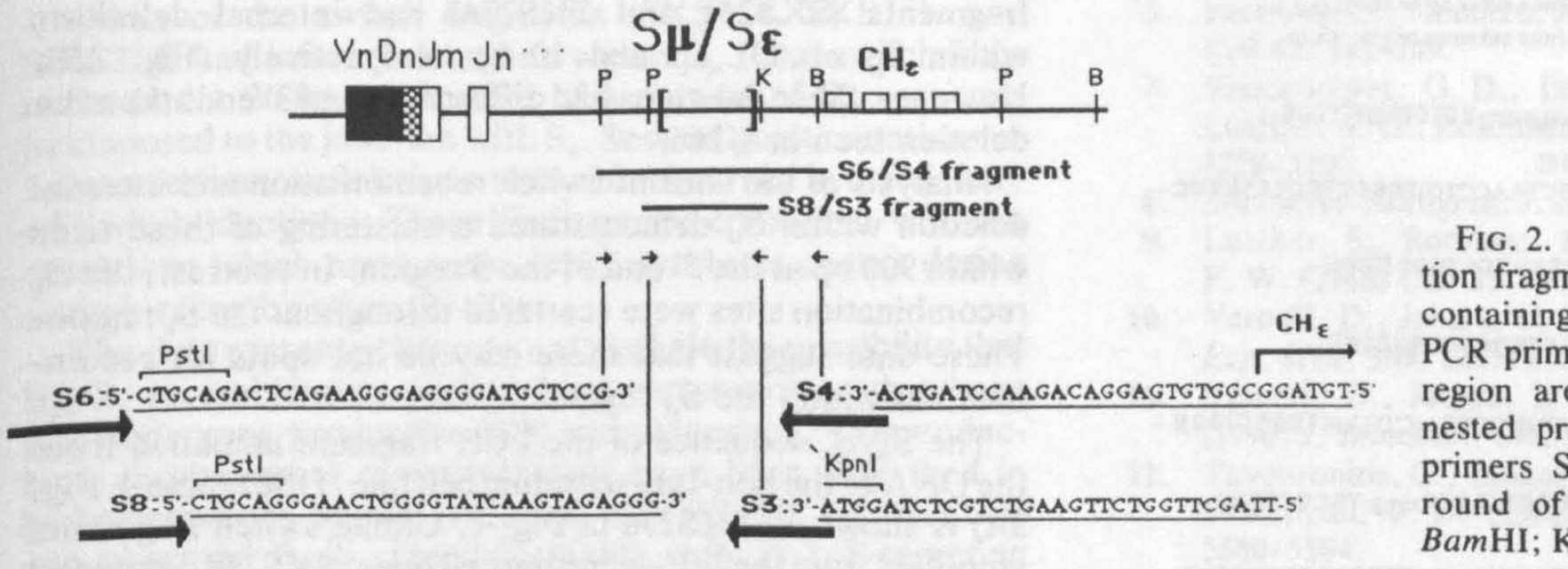

FIG. 2. PCR amplification of S_μ/S_ε junction fragments. A representative fragment containing an S_μ/S_ε junction is shown. The PCR primers used for amplification of the region are pictured below the map. For nested primer PCR, the first round uses primers S6/S4 while the second (nested) round of PCR uses primers S8/S3. B, *Bam*HI; K, *Kpn* I; P, *Pst* I.

PCR Amplification and Sequencing of the Switch Fragment from U266. Genomic DNA from the U266 cell line was subjected to one round of PCR amplification utilizing the primers S6/S4 or S8/S3 (Fig. 2). Amplified fragments were subcloned and DNA sequencing showed that a complex rearrangement had occurred between S_μ and S_ε regions. A 243-bp internal deletion occurred within S_μ, followed by an additional 186 bp of S_μ sequence (Fig. 3*B*). At the termination of the S_μ sequence, there was a 34-bp insertion segment, not homologous to either S_μ or S_ε (Fig. 4). Part of this insertion segment contained pentameric repeats typical of S region sequences (26, 27). However, comparison of this insertion segment with S_μ, S_ε, and $S_{\gamma 4}$ sequences (28) failed to reveal an unambiguous origin for this insert. The 34-bp insertion segment was ligated to an S_ε sequence that continued downstream without interruption into the IgE heavy chain gene coding region, $C_{H\varepsilon}$ (Fig. 3*B*).

The sequence of the fragment amplified by PCR from U266 DNA was identical to the sequence of a fragment isolated from a U266 genomic library that encompassed the S_μ/S_ε junction (unpublished data). Thus, PCR-amplified switch fragments accurately reflect the switch rearrangement in genomic DNA.

Nested-Primer PCR Amplification of Switch Fragments from IgE-Secreting B-Cell Lines. Because <5% of B cells in our cell lines contained cytoplasmic IgE, we tested the sensitivity of the PCR amplification method used above. Various dilutions of U266 DNA were mixed with fibroblast genomic DNA such that the final concentrations of U266 DNA in the mixtures were 20%, 2%, 0.2%, 0.02%, and 0.002%. The final dilution represented the DNA equivalent of two to three U266 cells in a 1-μg sample of fibroblast DNA. When PCR was performed utilizing the primers S6/S4 (Fig. 2), no PCR-amplified fragments were identified by gel elec-

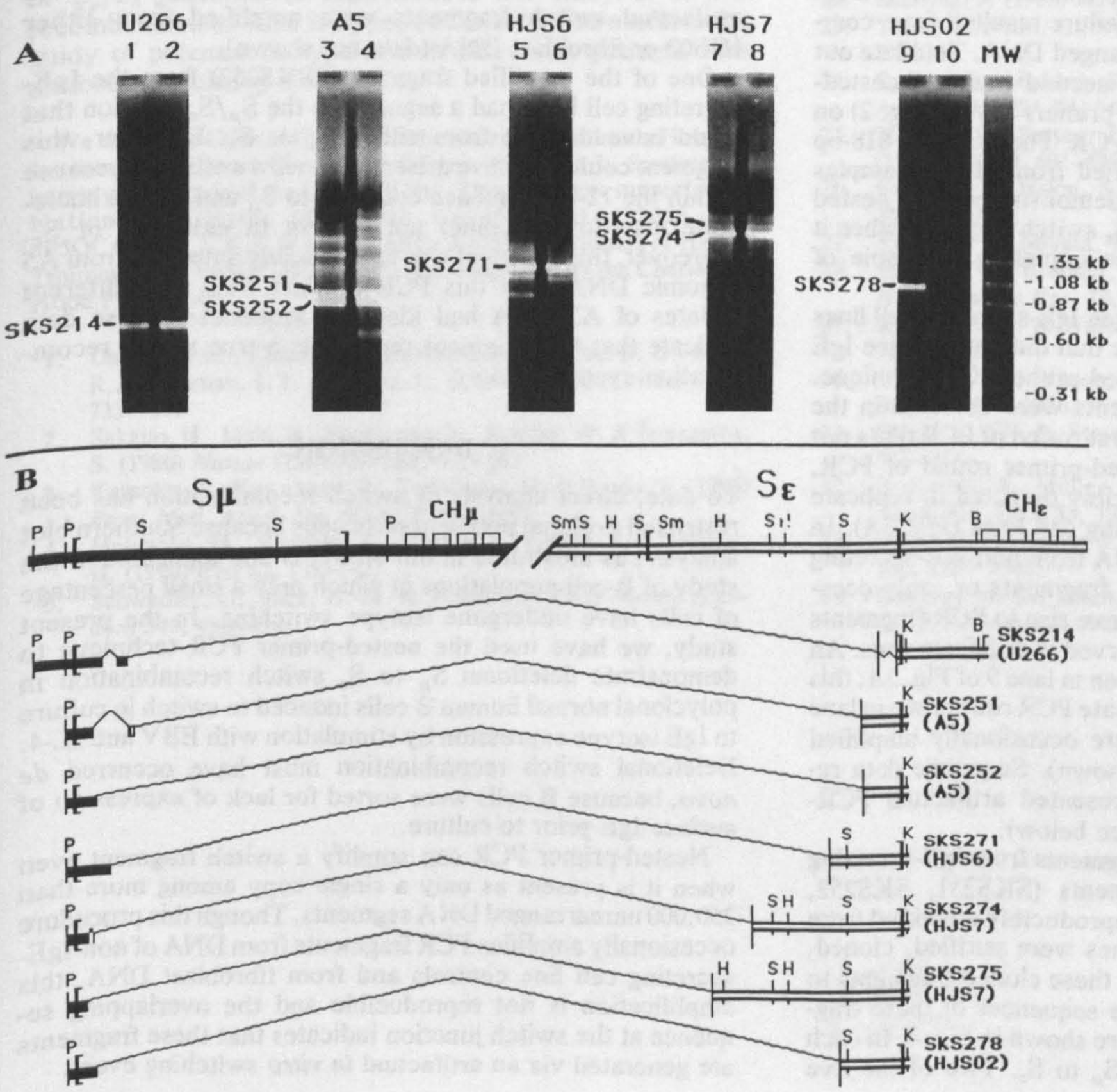

FIG. 3. Nested primer PCR products of U266, IgE-secreting (A5, HJS6, and HJS7), and non-IgE-secreting (HJS02) lymphoblastoid lines. (*A*) Agarose gel electrophoresis of PCR products. Each pair of samples represents two independently run nested-primer PCR products from the same genomic DNA preparation. The indicated fragments were isolated, cloned in plasmid vectors, and sequenced. (*B*) Diagrammatic summary of the sequenced DNA fragments encompassing the S_μ/S_ε junction. The genomic map of the unrearranged S_μ and S_ε regions is compared with the switch fragments isolated from U266 and the EBV B-cell lines. Three fragments (SKS214, SKS251, and SKS274) have internal deletions of S_μ. One fragment (SKS214) has an insertion segment of unclear origin joining S_μ to S_ε (represented as W). One fragment (SKS251) has a 26-bp duplication of a portion of S_μ at the 3′ end of the S_μ sequence. One example of an unreproducibly amplified fragment from the non-IgE-secreting cell line (HJS02) is shown in lane 9; this fragment is not present in the replicate sample shown in lane 10. Brackets represent the probable extent of the switch regions. B, *Bam*HI; H, *Hin*dIII; K, *Kpn* I; P, *Pst* I; S, *Sac* I; Sm, *Sma* I.

Sequence from Sμ: G,A,T,C
Sequence from either Sμ or Sε: G,A,T,C
Sequence from Sε: G,A,T,C
Sequence from unknown origin: g,a,t,c

SKS214 (U266):
···CTTAGCTGGGcTGAGttgggctgggctgagctgggctaagctggacctgACTGTGATTTTGG···

SKS251 (A5): *[Sμ Duplication bp 888-914]*
···CTAACCTGGGC[AGAGCTGAGCTGGGCTGAGCTAACCTG]TGGCCTGGCCTCGGC···

SKS252 (A5):
···CTGGGCTGAGCTGGGCAGGaCTGGGCTGAGCAGGCCTGAGCAGG···

SKS271 (HJS6):
···CTGAGCTGGGCTGAGCTGGGCTGAAATGGGCTGAGGTGAGCTAG···

SKS274 (HJS7):
···CTGCAGTAA-CTGGGCTGAGCTGGGCAGGGCTGGGCTGAaCTGAaCTGGGCTGGA···

SKS275 (HJS7):
···TTTCAGAAATGGACTCAGATGACCTGGCCTGAGTTCAGCagggcTGCGCTGAG···

SKS278 (HJS02):
···TTGGCTGCACTAAGCTGGGCTGGGCTGGGCTGGGCTGGGTTCAGCTGAGCGGG···

SKS277 (Fibroblast):
···CTGAGCTGGGCTGGGCTGAGCTGGGCTGGGCTGGGTTCAGCTGAGCGGGGTT···

FIG. 4. Sequence of the S_μ/S_ε switch junction in the cloned fragments. The DNA sequence data encompassing the S_μ/S_ε switch junction of the fragments in Fig. 3*B* are shown. The key at the top indicates origins of the nucleotides. Nucleotides that could originate from either S_μ or S_ε generally encompass pentameric repeat sequences that are common to both switch regions. The presence of sequences common to both S_μ and S_ε as in fragments SKS277 and SKS278 is indicative of *in vitro* artifactual switching. The common sequence in the SKS252 fragment is interrupted by a nucleotide not present in either switch region, thus suggesting that this fragment is not artifactual. The origin of the insertion segment in SKS214 is not known. The 26-bp duplicated segment in SKS251 is identical to bp 888–914 and is shown between brackets within the sequence. The hyphen in the SKS274 sequence represents a 1-bp deletion. The sequence of unknown origin in SKS275 within the S_ε region may not be a true insertion/deletion but may represent a polymorphism.

trophoresis, even when 20% of the template DNA was from U266. We hypothesized that this failure resulted from competition for the primers by unrearranged DNA. To dilute out this competition, we performed a second round of nested-primer PCR amplification utilizing primers S8/S3 (Fig. 2) on an aliquot from each first round of PCR. The expected 618-bp U266 switch fragment was amplified from all the samples (data not shown). These results demonstrated that nested primer PCR could amplify an S_μ/S_ε switch fragment when it was present at only two or three copies in a sample of unrearranged DNA.

Genomic DNA samples from three IgE-secreting cell lines (A5, HJS6, HJS7) and one cell line that did not produce IgE (HJS02) were amplified by the nested-primer PCR technique. As expected, no amplified fragments were detected in the DNA from the cell lines after the first round of PCR (data not shown). However, after the nested-primer round of PCR, multiple fragments were reproducibly detected in replicate samples from all three IgE-secreting cell lines (Fig. 3*A*). In contrast, PCR amplification of DNA from non-IgE-secreting cell lines revealed either no PCR fragments or, only occasionally (<30% of all PCR runs), gave rise to PCR fragments that were never reproducibly observed in duplicate runs. An example of one such fragment is seen in lane 9 of Fig. 3*A*; this fragment was not seen in the duplicate PCR run shown in lane 10. Similarly, PCR fragments were occasionally amplified from fibroblast DNA (data not shown). Sequence data revealed that these fragments represented artifactual PCR-generated "*in vitro* switching" (see below).

Sequence Analysis of Switch Fragments from IgE-Secreting B-Cell Lines. Five switch fragments (SKS251, SKS252, SKS271, SKS274, and SKS275) reproducibly amplified from DNA of the IgE-secreting cell lines were purified, cloned, and sequenced. Fig. 3*B* compares these cloned fragments to the genomic S_μ and S_ε maps. The sequences of these fragments around the S_μ/S_ε junction are shown in Fig. 4. In each case there was direct joining of S_μ to S_ε. Two of the five fragments (SKS251 and SKS274) had internal deletions within S_μ of 551 bp and 10 bp, respectively (Fig. 3*B*). However, these deletions had different 5′ and 3′ ends than the deletion seen in U266.

Analysis of the sites of switch recombination and internal deletion within S_μ demonstrated a clustering of these sites within 900 bp at the 5′ end of the S region. In contrast, the S_ε recombination sites were scattered throughout the S_ε region. These data suggest that there may be hot spots for recombination within the S_μ region.

The S_μ/S_ε sequence of the PCR fragment amplified from the DNA of the non-IgE-secreting cell line HJS02 (lane 9, Fig. 3*A*) is shown as SKS278 in Fig. 4. Unlike switch fragments amplified from the IgE-secreting cell lines, SKS278 contained at the junction between S_μ and S_ε a 9-bp sequence that is common to S_μ and S_ε regions and, therefore, could have derived from either S region. Allowing for two mismatches, the sequence in SKS278 common to both S regions would be 26 bp. Switch fragment SKS278 likely represents an *in vitro* artifact that arose because single-stranded PCR products bound to each other due to complementarity at their 3′ ends. Elongation of the annealed product resulted in amplification of the fragment leading to artifactual *in vitro* switching. The sequence of the switch junction of an artifactual PCR fragment amplified from fibroblast genomic DNA (SKS277) similarly contains a segment at the S_μ/S_ε junction (16 bp) that is common to the two S regions. We have confirmed the presence of the switch fragments in the IgE-secreting cell lines and the artifactuality of fragments amplified from HJS02 and fibroblast DNA by using another pair of primers (S7 and S9) for the nested-primer round of PCR. Switch fragments of the expected sizes were amplified with these primers from DNA of the IgE-secreting cell lines. In contrast, none of the artifactual switch fragments were amplified from either HJS02 or fibroblast DNA (data not shown).

One of the amplified fragments (SKS252) from the IgE-secreting cell lines had a segment at the S_μ/S_ε junction that could have derived from either S_μ or S_ε. However, this fragment could not have arisen by *in vitro* switching because within the 12-bp sequence common to S_μ and S_ε is a nucleotide insertion (adenine) not present in either S_μ or S_ε. Moreover, this fragment was reproducibly amplified from A5 genomic DNA, and this PCR product from two different isolates of A5 DNA had identical sequences. These data indicate that this fragment represents a true switch recombination event.

DISCUSSION

To date, direct analysis of switch recombination has been restricted to clonal populations of cells because Southern blot analysis, as illustrated in our study, is not applicable to the study of B-cell populations in which only a small percentage of cells have undergone isotype switching. In the present study, we have used the nested-primer PCR technique to demonstrate deletional S_μ to S_ε switch recombination in polyclonal normal human B cells induced to switch in culture to IgE isotype expression by stimulation with EBV and IL-4. Deletional switch recombination must have occurred *de novo*, because B cells were sorted for lack of expression of surface IgE prior to culture.

Nested-primer PCR can amplify a switch fragment even when it is present as only a single copy among more than 250,000 unrearranged DNA segments. Though this procedure occasionally amplifies PCR fragments from DNA of non-IgE-secreting cell line controls and from fibroblast DNA, this amplification is not reproducible and the overlapping sequence at the switch junction indicates that these fragments are generated via an artifactual *in vitro* switching event.

Three of the switch fragments (SKS214, SKS251, and SKS274) had internal deletions within S_μ. One switch fragment had a 26-bp duplication of a portion of S_μ sequence juxtaposed to the junction with S_ε. Several point mutations or 1-bp insertions or deletions were also observed in the vicinity of switch junctions. These findings are consistent with the models in which error-prone DNA synthesis occurs during switch recombination (29–31).

The data presented here do not exclude the possibility that S_μ/S_ε recombination occurred on chromosomes that have not undergone productive VDJ rearrangement. Nonproductive chromosomal rearrangements have been described in hybridomas and normal murine B cells (32–34). The fact that we observed S_μ/S_ε rearrangements only in IgE-secreting lymphoblastoid cells, and not in the non-IgE-secreting controls, strongly suggests that S_μ/S_ε switch fragments have the necessary upstream VDJ regions for productive IgE expression. The recent observation that an EBV-transformed B-cell clone has deleted $C_{H\mu}$ on both chromosomes indicates that S_μ/S_ε switching occurs on chromosomes that have productively rearranged their VDJ regions (17).

Our observations of S_μ/S_ε deletional switch recombination are in contrast to the results of MacKenzie and Dosch (15), who failed to detect S_μ/S_ε recombination by Southern blot analysis of IgE-secreting human B-cell clones. The discrepancy may be due to different modalities used to induce IgE synthesis. MacKenzie and Dosch cultured unsorted B cells in the presence of EBV and irradiated T-T hybridoma filler cells in the absence of exogenous IL-4. Nonetheless, our results cannot exclude nondeletional mechanisms of IgE isotype switching.

The approach we have used to study the mechanism of isotype switching to IgE by nested-primer PCR amplification of S_μ/S_ε junctions is applicable to the study of switch recombination to other isotypes and could prove useful in the study of potential isotype switch defects in patients with antibody deficiency syndromes.

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